ADENINE NUCLEOTIDE TRANSLOCATION IN RAT LIVER MITOCHONDRIA: CALCIUM STIMULATION AND THE INVOLVEMENT OF PHOSPHOLIPIDS

by

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STATEMENT

This thesis describes the results of a study conducted in the Department of Biochemistry, School of General Studies at The Australian National University, Canberra, under the supervision of Doctor F.L. Bygrave.

All work was carried out by myself with the exception of some portions of that described in Sections D and E where technical assistance was rendered by Ms A. Daday and the electron-micrographs which were produced by Mr M. McCuaig.

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SUMMARY

Ca^{2+} ions were found to stimulate the translocation of ATP, and to a lesser extent, ADP by isolated rat liver mitochondria. Studies using a variety of parameters implicate the low affinity Ca^{2+} binding sites on the outside of the inner mitochondrial membrane in this stimulatory process. The stimulation by Ca^{2+} ions is independent of the energy status of the mitochondrion (the stimulatory effects of CCCP and Ca^{2+} on ATP translocation are additive) and occurs by a mechanism which differs from that whereby K^{+} and Mg^{2+} produce a similar, if somewhat smaller, increase in the rate of translocation. In the presence of Ca^{2+} ions ATP is able to effectively compete with ADP for translocation, a point which re-enforces the hypothesis that Ca^{2+} has a potential for modifying reactions involved in the regulation of cell metabolism.

Local anesthetics inhibit the translocation of ATP and ADP; the most effective is butacaine. However, at both low butacaine and adenine nucleotide concentrations butacaine was observed to stimulate the rate of translocation. This and other properties of butacaine with regard to adenine nucleotide translocation were investigated. That portion of ATP translocation stimulated by Ca^{2+} was preferentially inhibited by each of the local anesthetics tested. In contrast, inhibition by the anesthetics of ADP translocation was prevented by low concentrations of Ca^{2+}. Aliphatic alcohols were also capable of inhibiting the activity of the adenine nucleotide translocase. A variety of membrane-active agents were tested for their effects on ATP and ADP translocation in the absence and presence of Ca^{2+}. On the basis of the latter observations it is concluded that direct comparisons between the effect of these agents on mitochondrial membranes and nerve cell membranes are not necessarily valid.

Studies using mitochondria that had been partially depleted of their phospholipids indicate that adenine nucleotide translocation exhibits specificity for particular phospholipids. This is well illustrated when one compares translocation rates in pancreatic phospholipase A (cardiolipin preferentially hydrolysed)

and *Crotalus adamanteus* venom phospholipase A (specific for phosphatidylcholine and phosphatidylethanolamine). As a generalisation greater than five times more phospholipid must be hydrolysed by the latter enzyme to achieve comparable decreases in translocation rates. Control experiments support the contention that the loss in translocation activity is due to loss of phospholipid and not due to the effects of the hydrolysis products. Binding studies also indicate that there is a loss of the ability of the phospholipase A treated mitochondria to bind adenine nucleotides.

In the concluding section the control of adenine nucleotide translocation *in vivo* is discussed. Evidence is also tabulated and an hypothesis promoted whereby there exists more than one type of adenine nucleotide binding/translocation site in rat liver mitochondria.

PUBLICATIONS

'Stimulation by calcium of atractyloside-sensitive adenine nucleotide translocation in rat liver mitochondria', Spencer, T. and Bygrave, F.L., Biochem. Biophys. Res. Commun. 43, 1290 (1971).

'Modification by Ca^{2+} ions of adenine nucleotide translocation in rat liver mitochondria', Spencer, T. and Bygrave, F.L., Biochem. J. 129, 355(1972).

'The influence of Lanthanum on calcium-stimulated ATP translocation in rat liver mitochondria', Spencer, T. and Bygrave, F.L., FEBS Letters 26, 225 (1972).

'The influence of calcium ions on the atractyloside-sensitive translocation of adenine nucleotides by rat liver mitochondria', Spencer, T. and Bygrave, F.L., Proc. Aust. Biochem. Soc. 4, 97 (1971).

DEDICATION

To my late father

without whom

this thesis, nor the work embodied in it,

would not have evolved.

TABLE OF CONTENTS

SECTION A: INTRODUCTION - ADENINE NUCLEOTIDE TRANSLOCATION

1 General

The adenine nucleotide translocase is an enzyme carrier system whose specific function is to transport adenine nucleotides across the inner membrane of mitochondria. Although evidence for the existence of this carrier system has occurred only in the last decade already aspects of it have been studied in some detail. These will be discussed later in the introduction. The work embodied in this thesis involves a study of the following aspects of the adenine nucleotide translocase: (a) the interaction of metal ions, in particular Ca^{2+}, with the translocase, and (b) the role of phospholipid in the translocation process. Thus the introductory section of this thesis will deal not only with the known properties of adenine nucleotide translocation in mitochondria but also with structure-function relationships in the membranes of these organelles. Finally consideration is given to the role of the translocase in cellular metabolism.

2 Structure of mitochondrial membranes

Mitochondria exist in the cytoplasm of all eukaryotic cells. A number of features are common to those isolated from a variety of sources. These include the possession of an inner and outer membrane, the latter completely surrounding the mitochondrion in the form of a sac. The inner membrane is composed of a number of internal folds or projections called cristae which result in it having a greatly increased surface area. In general the metabolically-active mitochondria, such as those from heart and insect flight muscle, possess a larger number of these cristae than, for example, mitochondria from cells such as lymphocytes. This proliferation of the cristae is intimately associated with the principal function of these organelles which is to provide energy, in the form of ATP, to the cell by way of oxidative phosphorylation. Other mitochondria are specialised to some degree as exemplified by those from adrenal cortex which are capable of steroid hydroxylations (196).

The configuration of the cristae is dependent both on the functional state of the mitochondria (82,84,266) and the composition of the suspending medium (11,85). The space bounded by the inner membrane is the mitochondrial matrix or intracristal space which consists of a viscous protein gel. Suspended in the gel are electron-dense granules of unknown function, a variety of enzymes and enzyme-systems including all the apparatus needed for protein synthesis, a unique circular DNA, metabolites and ions. Between the inner and outer membranes is the intermembrane space which contains a small number of soluble enzymes.

3 Composition of mitochondrial membranes

Relatively 'pure' inner and outer membrane fractions may be isolated by the use of a variety of methods which strip off the outer membrane. These include digitonin treatment (20,102,220) and 'swelling/shrinking' (191). The former method utilises the property of digitonin which enables it to form insoluble complexes with chloesterol. This property provides evidence that cholesterol is chiefly localised in the outer membrane as the inner membrane preparations formed by this method remain virtually intact and are capable of oxidative phosphorylation and other membrane reactions (100,220). Insertion of cholesterol into 'black lipid membranes' and liposomes has been shown to make these artificial membranes more rigid (184). Similar effects presumably occur within the outer membrane and this property results in a protective area around the more essential part of the mitochondrion the inner membrane and matrix. The composition of purified inner and outer membranes prepared by the action of digitonin is shown in Table I (adapted from ref.45). Somewhat similar results are also obtained using the swelling/shrinking technique (45).

The following are the major features which distinguish the two types of membrane from each other: (a) the phospholipid/protein ratios vary by a factor of two, the inner membrane having the lowest value, (b) the cholesterol/phospholipid ratios vary by a factor of approximately 20, the inner membrane again having the lowest value, (c) the molar ratios of phosphatidylcholine:phosphatidylethanolamine:cardiolipin are 4:4:2 and 5:3:1 for the

TABLE I

Composition of purified inner and outer mitochondrial membranes prepared by digitonin treatment

Individual phospholipid compositions are expressed as % of total.

	Outer membrane	Inner membrane
Phospholipid/protein (moles/mg)	0.37	0.20
Cholesterol/phospholipid (mole/mole)	0.132	0.006
Lysophosphatidylcholine	2	1
Sphingomyelin	4	1
Phosphatidylcholine	46	38
Phosphatidylinositol	8	2
Phosphatidylethanolamine plus phosphatidylserine	31	38
Cardiolipin plus phosphatidic acid	9	20

inner and outer membranes respectively, and (d) outer membrane phosphatidylserine and sphingomyelin contents are four times higher than those of the inner membrane.

Lipid molecules possess a greater energy of interaction when they contain saturated chains (219). The higher rigidity of the outer membrane can be attributed not only to the high cholesterol content but also to its saturated/unsaturated fatty acid composition as shown in Table II (adapted from 45).

The inner membrane of the mitochondrion is the most specialised of the two and contains, embedded in it, a variety of enzymes including the complex necessary for oxidative phosphorylation. Part of this complex is represented by the so-called 'elementary knobs' which are localised on the innermost or matrix side of the membrane (70). These spherical particles which are joined to the membrane proper by a narrow stalk are the mitochondrial ATPase (206) and provide evidence for an assymetric nature of this membrane. This feature is a necessary component of the 'Chemisomotic' theory of oxidative phosphorylation (173,175).

The assymetric nature of the inner membrane has been confirmed using a number of techniques including: (a) preparative free flow electrophoresis of outside-out and inside-out inner membrane vesicles (90), (b) protein labels and controlled peptidolysis (see ref.264 for techniques). Schneider *et al* (221) suggest that both cytochrome a and cytochrome c face the outer membranes, while cytochrome a_3 and ATPase are on the matrix side; cytochrome oxidase is located on both sides of the inner membrane, (c) transportation or binding studies with ions or substrates (140,155,161,174), (d) freeze etching (276), and activities of side specific enzymes (181).

4 Mitochondrial permeability

Investigations of the permeability properties of whole mitochondria (120,181) have revealed the following points: (a) 60% of the intramitochondrial volume is permeable to a great number and variety of low molecular weight substances, e.g. glycerol, certain ions and nucleotides, when suspended in isotonic sucrose, (b) this volume correlates well with the intracristal space or matrix as seen by electron microscopy, (c) this space is

TABLE II

Saturated/unsaturated fatty acid ratios of mitochondrial phospholipids

Phospholipid	Outer membrane	Inner membrane
Total	1.20	0.72
Phosphatidylcholine	0.94	0.69
Phosphatidylinositol	0.47	not determined
Phosphatidylethanolamine	1.07	0.85
Cardiolipin	not determined	0.12

not accessible to larger molecules such as sucrose, (d) the size of the space that is accessible to sucrose can be influenced by the tonicity of the suspending medium; under hypotonic conditions the sucrose inaccessible space increases and the matrix space swells as indicated by electron microscopy. Thus the sucrose-accessible space is identical with the intermembrane space and the sucrose-inaccessible space with the matrix space. Essentially the inner membrane is osmotically active and behaves as an osmometer.

Other observations on the permeability of the mitochondrion indicate that adenine but not pyridine nucleotides are able to enter the matrix space and that the equilibration of these adenine nucleotides is slow. Entry and exit of adenine nucleotides by free diffusion was found to be unlikely since isolated mitochondria contain a relatively large amount of adenine nucleotides (119). On the basis of this type of information it was proposed that the permeation of adenine nucleotides across the mitochondrial inner membrane was dependent on and facilitated by an enzyme or enzyme system later designated the adenine nucleotide translocase (92,198).

Properties of the adenine nucleotide translocase

1 General

Experiments conducted in several laboratories on the translocation of adenine nucleotides across the mitochondrial membrane have revealed a number of features about the process. It is specific for ATP and ADP and their deoxy and phosphoric acid analogues (62,199,270). AMP may be translocated but not as the intact molecule. It is first converted to ADP in the presence of small amounts of ATP, which may have 'leaked' out of the mitochondrion, by adenylate kinase located in the intermembrane space. Adenosine, adenine as well as guanine, uridine and inosine mono-, di- and triphosphates are also inactive as substrates. They may, however, be transported in a very slow process which has been shown to be simple diffusion.

One compound which has contributed largely to investigations carried out on the function and properties of the adenine nucleotide translocase is atractyloside. The finding that this

substance is able to specifically inhibit the obligatory exchange of exogenous and endogenous adenine nucleotides greatly substantiates the hypothesis that this process is catalysed by a specific translocase enzyme. Historical aspects of the elucidation of the role and properties of the adenine nucleotides translocase may be obtained in references 22,23,40,41,61,116,254.

2 Kinetics

Analysis of the time course kinetics of the translocation of ATP and ADP have indicated that the reaction is first order with respect to the size of the endogenous exchangeable adenine nucleotide pool. The first order rate constant is inversely proportional to the size of this pool (121,122,201). Souverijn *et al* (239) have questioned this observation. They have shown that the exchange of ATP, but not of ADP, with endogenous adenine nucleotides is biphasic and dependent on the endogenous ATP/ADP ratio. Evidence is also presented by them which indicates that the rapid initial phase of the ATP exchange is an electroneutral exchange of external ATP with internal ATP.

Studies of the substrate kinetics indicate that the translocase is half-saturated at 1.3μM ADP and 2.5μM ATP (201). These values were obtained using low protein concentrations, <0.1mg per ml. When the protein concentration is increased by an order of magnitude apparent K_m values of up to 220μM for ATP and 48μM for ADP have been reported (165). Under standard conditions, i.e. with coupled mitochondria, ADP is always translocated 2–3 times faster than ATP (199,301). An interesting feature of the 'Eadie' plots used by Pfaff *et al* to determine the affinity of the translocase for ADP and ATP was the non-linearity of these plots (201). These workers suggest that this may indicate the presence of another group of translocation sites in mitochondria possessing a lower affinity for adenine nucleotides. Recent work (239) has indicated that the K_m for ATP is dependent on the energy state of the mitochondrion; values for K_m vary from 1.5μM in the presence of uncoupler (low energy conditions) to 200μM under high energy conditions (in the presence of 10mM succinate). The K_m for ADP, however, is unaffected by changes in the energy state of the mitochondria.

Differences between the rate of translocation of ATP and ADP are most pronounced when the adenine nucleotides are added simultaneously to the mitochondria (121,199). Competition occurs between ATP and ADP such that ADP is translocated at a rate of up to ten times faster than that of ATP. This large difference in translocation specificity is not observed with the concomitant exit of adenine nucleotides from the mitochondria. ATP and ADP are released in proportion to their intramitochondrial content, i.e. the affinity of the translocase on the inside of the mitochondrion is the same for both ATP and ADP (199).

3 Electrogenic nature

The rate of translocation of ATP and consequently its ability to compete with ADP as a substrate is influenced by the metabolic state of the mitochondrion. Thus when mitochondria are uncoupled, by e.g. CCCP, the translocation rate of ATP is increased and that of ADP decreased slightly such that they are equivalent (121,199,239). Under these circumstances the preference for ADP as substrate is lost. The process is independent of the mitochondrial ATPase as indicated by the fact that the effect is oligomycin-insensitive. This is substantiated by the K_m values for stimulation of ATP translocation and stimulation of mitochondrial ATPase by CCCP which are 0.08μM (199) and 5μM (94) respectively, over fifty-fold different from each other.

On the basis of these results it has been suggested that, basically, the translocation process has equal specificities for ADP and ATP and only when the mitochondria are coupled or in the control state is ADP translocated preferentially (121,122). Fig. 1 shows a scheme which has been proposed by Klingenberg *et al* to explain this phenonemon (121). At neutral pH, ATP and ADP exist as the ATP^{4-} and ADP^{3-} species. Translocation of ADP into the mitochondrion occurs with little resistance as the electrical equilibrium can be maintained by the exit of ADP^{3-}. Entry of ATP on the other hand results in a build-up of OH^- ions and a subsequent 'back-pressure' of negative charge which inhibits further ATP translocation unless there is a concomitant influx of H^+ ions. Uncouplers facilitate the latter operation by breaking down the membrane potential and allowing the entry of H^+ to neutralise the OH^- ions consistent with the 'Chemiosmotic'

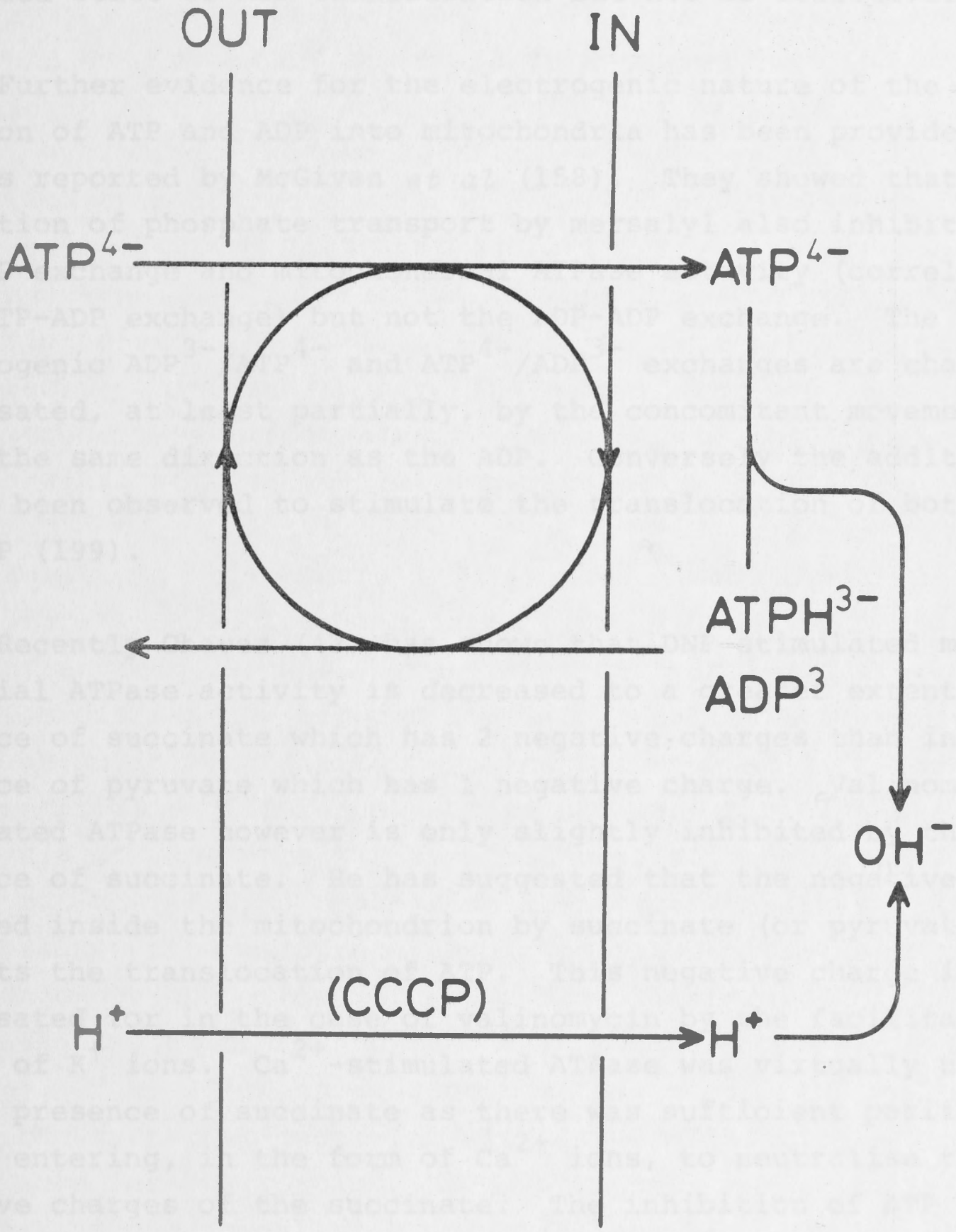


Figure 1. *Mechanism for inhibition of the translocation of exogenously added ATP by mitochondria and the release of inhibition by uncouplers.*

Data essentially according to Klingenberg and Pfaff (121).

hypothesis of Mitchell (173,175). Valinomycin which promotes the entry of K^+ into the mitochondrion is also able to overcome the inhibited state of ATP translocation but not as effectively.

Further evidence for the electrogenic nature of the translocation of ATP and ADP into mitochondria has been provided from results reported by McGivan *et al* (158). They showed that the inhibition of phosphate transport by mersalyl also inhibited the ADP-ATP exchange and mitochondrial ATPase activity (correlated with ATP-ADP exchange) but not the ADP-ADP exchange. The electrogenic ADP^{3-}/ATP^{4-} and ATP^{4-}/ADP^{3-} exchanges are charge compensated, at least partially, by the concomitant movement of Pi in the same direction as the ADP. Conversely the addition of Pi has been observed to stimulate the translocation of both ADP and ATP (199).

Recently Chavez (42) has shown that DNP-stimulated mitochondrial ATPase activity is decreased to a greater extent in the presence of succinate which has 2 negative charges than in the presence of pyruvate which has 1 negative charge. Valinomycin-stimulated ATPase however is only slightly inhibited by the presence of succinate. He has suggested that the negative charge produced inside the mitochondrion by succinate (or pyruvate) inhibits the translocation of ATP. This negative charge is partly compensated for in the case of valinomycin by the facilitated influx of K^+ ions. Ca^{2+}-stimulated ATPase was virtually unchanged in the presence of succinate as there was sufficient positive charge entering, in the form of Ca^{2+} ions, to neutralise the negative charges of the succinate. The inhibition of ATP translocation has been confirmed by Souverijn *et al* as being due to a decreased affinity for ATP in the presence of succinate (239).

It can be concluded from these findings that the preferential specificity for adenine nucleotide translocation requires the consumption of energy. This energy is supplied by the respiratory chain. It has not yet been established in detail how the transport process is influenced by the energy. It is assumed that through the transport of electrons by the respiratory chain a potential is built up across the inner membrane, negative with respect to the inside, which allows a discrimination between the differently charged ADP and ATP species (see ref.122).

4 Temperature Sensitivity

Adenine nucleotide translocation is a highly temperature-sensitive process. In the case of ATP, the translocation rates are 2 and 80nmol/mg/min at 0° and 20°C respectively, a forty-fold increase in 20°C(201). Arrhenius plots of translocation activity exhibit a biphasic character. Pfaff *et al* have reported that the transition temperature occurs at 8°C with activation energies of 34-35 Kcal below and 21-23Kcal above 8°C for both ATP and ADP(201). Duée and Vignais assign the temperature break to 10°C with activation energies for ADP of 49Kcal below and 13Kcal above this value (62). This high temperature sensitivity has important consequences when mitochondrial adenine nucleotide requiring reactions are studied at low temperatures.

Heldt and Klingenberg (93,94) have observed that at low temperatures, uncoupler-stimulated hydrolysis of exogenous ATP was much faster than the equivalent rates of hydrolysis of exogenous ATP. The discrepancy between the two was alleviated to some extent by an increase in temperature but even at 25°C there still was a noticeable difference. Hence the rate limiting step in the hydrolysis of exogenous ATP by mitochondria is not the ATPase itself but the adenine nucleotide translocase. Similar differences were obtained from measurements of the phosphorylation of exogenous and endogenous ADP. However above approximately 14°C the phosphorylation reaction and not ADP translocation becomes rate limiting. The different susceptibilities of the two reactions is due to the fact that uncoupler-stimulated ATPase is up to 5 times faster than phosphorylation; ATPase is only a partial reaction of oxidative phosphorylation and thus the rate limiting step in the latter probably involves a reaction located before the ATP synthetase step. One other explanation is that the forward (ATP synthetase) and backward reactions (ATPase) do not proceed at the same rates.

Translocation of both ATP and ADP is virtually unaffected by changes in the pH of the suspending medium between 5.0 and 8.5 (199). Slight optima were observable at pH 7.0 for ADP and 7.6 for ATP.

5 Inhibitors

Atractyloside is a competitive inhibitor of adenine nucleotide translocation in mitochondria isolated from animals and yeast but not plants (for review see refs.95,127,128,192). This compound is a plant glycoside derived from the rhizomes of the mastic thistle (Atractylis gummifera). The probable structure of atractyloside is shown in Fig. 2. It consists of a diterpene moiety, atractyligenin , linked by a glycosidic bond to a molecule of D(+) glucose which has only one free hydroxyl group (C-6'), the C-2' hydroxyl group being linked to isovaleric acid and the C-3' and C-4' hydroxyl groups to sulphuric acid (203).

Another compound called carboxyatractyloside (gummiferin) which is also an inhibitor of adenine nucleotide translocation, has been isolated from 'Atractylis' (51,53,240,256). It differs from atractyloside in that it has a second carboxyl group at C-4 which gives it a total of 4 net negative charges. Studies of Defaye *et al* have shown it to be a natural precursor of atractyloside (54).

Recently a third compound with atractyloside-like properties has been identified and isolated from extracts of Atractylis gummifera (211,218). This compound is identical to epi-atractyloside, an isomer of atractyloside, in which the C-4 carboxyl group is equatorial instead of axial. Like carboxyatractyloside epi-atractyloside has a higher affinity for mitochondrial membranes than atractyloside itself. This observation offers an explanation for the higher affinity of carboxyatractyloside and epi-atractyloside for the translocase as both have in common the equatorial carboxyl group which in space-filling models is very close to the two $-SO_3^-$ groups on the glucose moiety. Thus the configuration of the anionic charges, as opposed to the number of charges, is the important determinant of the binding capabilities.

Atractyloside derivatives which inhibit ADP-stimulated respiration but to a lesser extent than atractyloside itself include apo-atractyloside which has a free hydroxyl group at C-2', atractyligenin, which lacks the glucose moiety and atractyloside diacetate which has acetate groups attached to the C-6' and C-6' and C-15 hydroxyl groups (259). The importance of the double bond

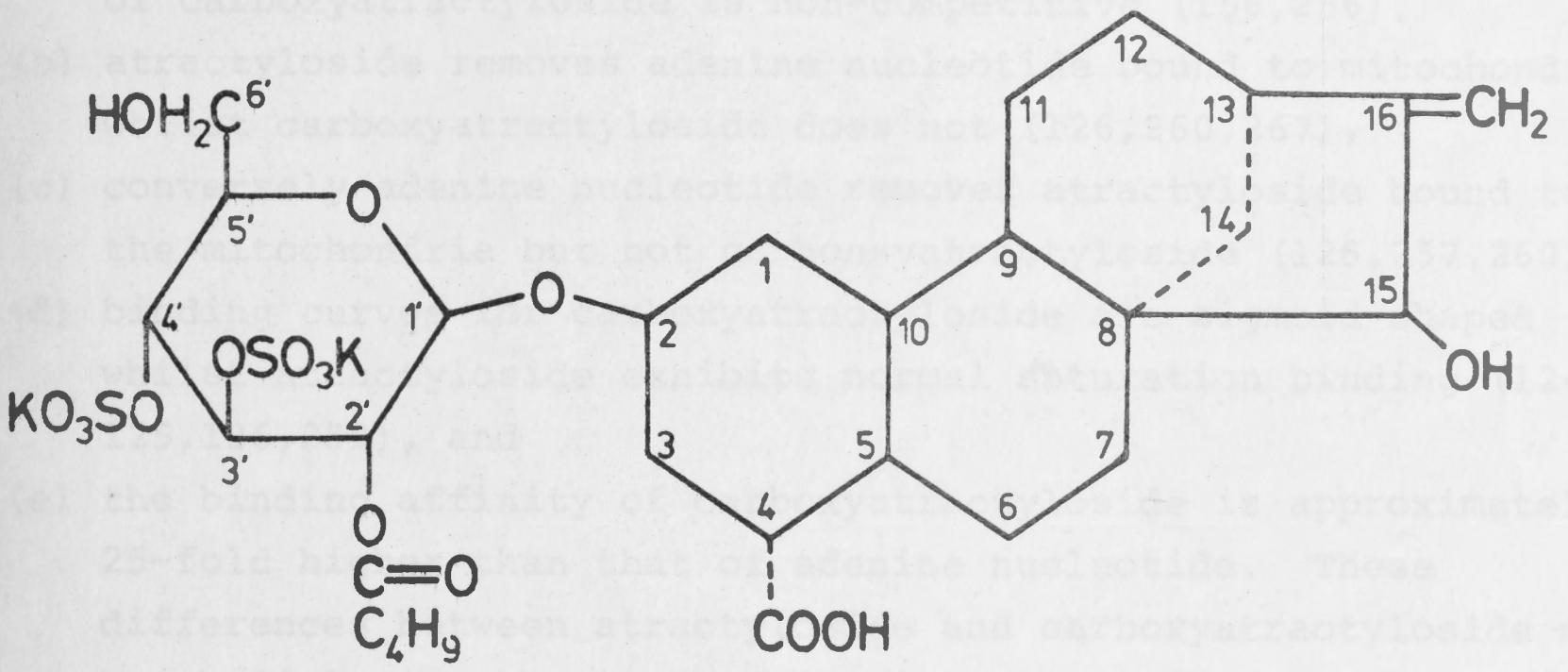


Fig.2. Structure of atractyloside

at C-16/C-17 is indicated by the observation that hydrogenation virtually leaves the atractyloside inactive (210).

The addition of the extra carboxyl group to carboxyatractyloside confers several properties on this compound which differ from those of atractyloside. These include:

(a) atractyloside inhibition of the translocase is competitive, (i.e. can be overcome by excess adenine nucleotide whilst that of carboxyatractyloside is non-competitive (156,256),
(b) atractyloside removes adenine nucleotide bound to mitochondria whilst carboxyatractyloside does not (126,260,267),
(c) conversely adenine nucleotide removes atractyloside bound to the mitochondria but not carbonxyatractyloside (126,257,260),
(d) binding curves for carboxyatractyloside are sigmoid-shaped whilst atractyloside exhibits normal saturation binding (124, 125,126,259), and
(e) the binding affinity of carboxyatractyloside is approximately 25-fold higher than that of adenine nucleotide. These differences between atractyloside and carboxyatractyloside may be abolished by the loss of the isovaleric acid residue from the C-2' residue to form apo-carboxyatractyloside (260).

Similarities between the interactions of atractyloside and carboxyatractyloside include:

(a) atractyloside binding is competitively inhibited by carboxy-atractyloside and vice versa (260), and
(b) both atractyloside and carboxyatractyloside binding are non-competitively inhibited by bongkrekic acid (125,126,260).

Bongkrekic acid (Figure 3) is an antibiotic isolated from cultures of *Pseudomonas cocovenans* which grows on rotting coconuts (253). This compound also inhibits the translocation of adenine nucleotide across the mitochondrial membrane (98,123) but in a manner different to that of both atractyloside and its derivatives. It exerts its effects by increasing the binding of adenine nucleotides to the mitochondrial translocase (66,126). In the case of ADP the affinity for ADP is increased 25-100-fold in the presence of bongkrekic acid. Thus the binding of adenine nucleotides to the translocase enzyme becomes virtually irreversible and therefore leads to inhibition of translocation as the enzyme is unable to release the adenine nucleotide. Unlike atractyloside whose effects are instantaneous the inhibition by

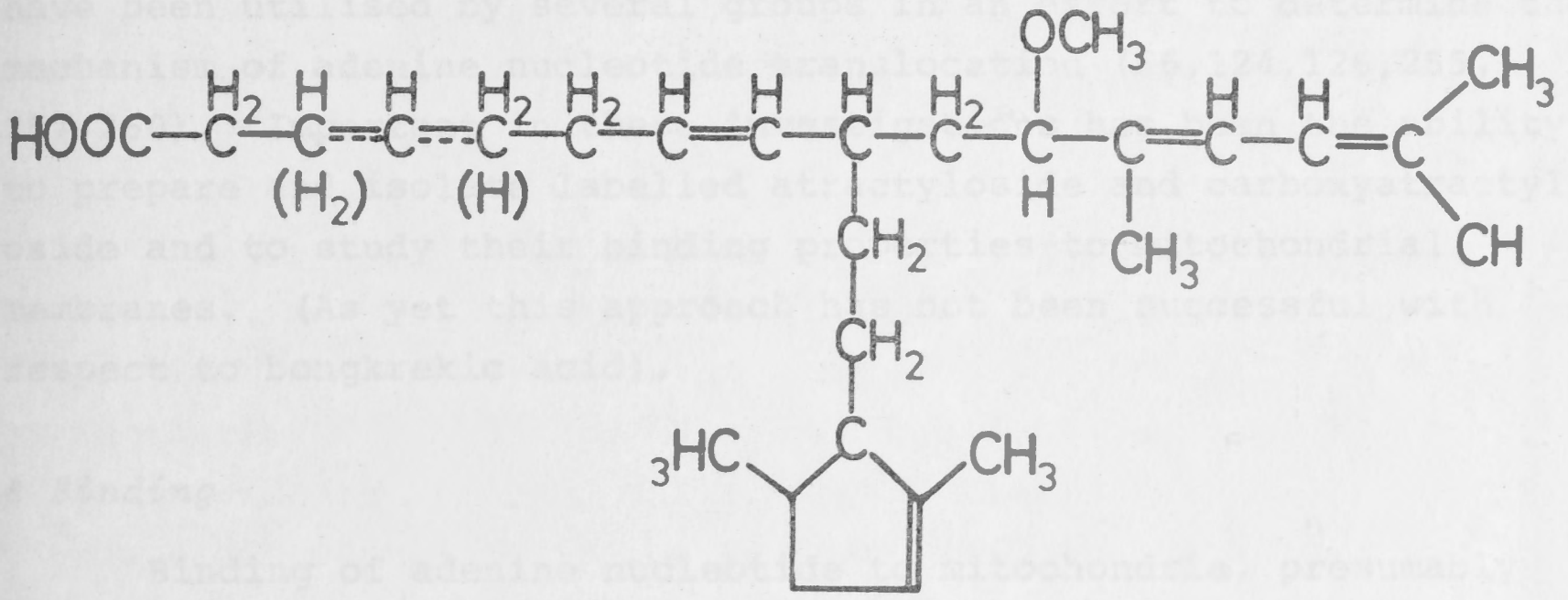


Fig. 3. Structure of bongkrekic acid

bongkrekic acid exhibits a time lag whose duration may be shortened by raising the temperature or prior incubation with adenine nucleotide (66,126). Erdelt *et al* (66) have explained this phenomenon as being due to a need for the inhibitor to diffuse across the mitochondrial membrane to exert its effects. Henderson and Shug (99) have suggested another alternative, on the basis of shortening of the time lag by approximately 10-fold in the presence of Coenzyme A, whereby the bongkrekic acid may first need to be converted to a CoA derivative before it can inhibit adenine nucleotide translocation.

Inhibitors of the adenine nucleotide translocation process have been utilised by several groups in an effort to determine the mechanism of adenine nucleotide translocation (66,124,126,255, 257,260). Important in these investigations has been the ability to prepare and isolate labelled atractyloside and carboxyatractyloside and to study their binding properties to mitochondrial membranes. (As yet this approach has not been successful with respect to bongkrekic acid).

6 Binding

Binding of adenine nucleotide to mitochondria, presumably at the site of adenine nucleotide translocase enzyme, has been investigated using mitochondria which have been depleted of their endogenous adenine nucleotide by a variety of methods. These include freeze-thawing, Pi treatment, arsenate treatment, detergent and digitonin treatment (66,127,128,260,267,271,272). Using preparations of this type, Weidemann *et al* (267) have assayed for binding activity on the basis that when the mitochondria are preloaded with labelled adenine nucleotide the addition of atractyloside would competitively remove that portion which is specifically bound at the translocase. By the use of this technique the following properties of this binding have been determined:

(a) rat liver mitochondria possess a single type of binding site, with a K_d for ADP of 0·5μM, which is present at 1·2 mole/mole cyt a. Rat heart mitochondria however possess two types of binding sites of unequal affinity, K_d's equal to 1μM and 4μM, with 2·2 sites per cyt a and a ratio of 1:4,

(b) binding is sensitive to methylene blue photo-oxidation and decreases between pH 7·0 and 7·5 suggesting histidine group involvement,

(c) disruption of mitochondria by sonication, lubrol, triton X-100 and deoxycholate treatment results in complete loss of atractyloside-removeable binding.

Winkler has found, however, that atractyloside prevents the binding of ADP to lubrol-treated mitochondria (271,272). This difference probably reflects the different types of binding assay employed. It may be that atractyloside prevents the binding of adenine nucleotide in lubrol-treated membranes but does not displace bound adenine nucleotide. This property is characteristic of the effect of carboxyatractyloside on adenine nucleotide binding (see above).

Using inner and outer membranes of mitochondria prepared by digitonin treatment, Winkler found a portion of the atractyloside-sensitive binding in the outer membrane fraction (272). This contamination was ascribed to shearing off of pseudopodal inner membrane processes formed during the digitonin treatment. Mainly on the basis of this evidence he suggests that the adenine nucleotide binding (translocation) sites are heterogenously distributed throughout the inner membrane with preference being given to the region in closest proximity to the outer membrane.

7 Models

Carrier models consistent with both the properties of adenine nucleotide translocation and binding have been proposed by Weidemann *et al* (267). The simplest of these is a dimer model which is compatible with the observations that endogenous adenine nucleotides are retained in the presence of exogenous adenine nucleotide and that binding sites of different affinities are present on the mitochondrion. The sites on the inner or matrix side of the membrane were assigned the low apparent K_d. They would be occupied with endogenous adenine nucleotide and thus would be saturated after addition of a low concentration of exogenous adenine nucleotide.

One problem with this model is that the high affinity sites comprise 50% of the total whereas experimental data places this figure closer to 20%. In light of this the model may have to be extended to either a tetramer as already suggested by Weidemann *et al* or two dimers in juxtaposition.

Further evidence for a multi-sited translocase comes from cooperative binding of carboxyatractyloside to mitochondria (256,260). By analogy to atractyloside this inhibitor acts at or near the adenine nucleotide translocase. Sonication, which most likely disrupts such oligmeric structures, results in the disappearance of this cooperative binding (260).

Role of the Adenine Nucleotide Translocase

The translocation process has a high affinity for both ATP and ADP. This affinity is much greater than the affinity of other enzymes reacting with these adenine nucleotides. The physiological significance of this is that under all circumstances the translocase is fully saturated both on the inside and outside of the inner mitochondrial membrane. It is hard to imagine conditions in the cell during which this situation would not be operative.

Occurrence of endogenous ADP in the control state is well explained by assuming a steady state equilibrium between endogenous adenine nucleotide and the respiratory chain (96). The fact that this ADP represents a relatively large proportion of the total adenine nucleotide reflects the relatively low endogenous phosphorylation potential required for the equilibration with the respiratory chain.

Heldt *et al* have determined the ATP/ADP ratios in the mitochondrial matrix and in the extramitochondrial space for mitochondria in an *in vitro* steady state system (97,122). The steady state ratio of endogenous ATP to ADP is the difference between the reversible first-order forward (ADP phosphorylation) and backward (ATP hydrolysis) reactions (96). Data in Table III compares the adenine nucleotide ratios and resultant free energies of phosphorylation in the mitochondrial matrix and in the extramitochondrial space for mitochondria in an *in vitro* steady state system.

TABLE III

Phosphorylation potentials of extramitochondrial and intramitochondrial compartments

$\Delta G' = \Delta G°' - 1.34 \log K$ where $\Delta G'$ = phosphorylation potential; $\Delta G°' = -8{\cdot}8$ Kcal/mol; K^+ = ATP/ADP.Pi; Pi (initial) = 0·5mM.

Compartment	ATP/ADP	Pi	$\Delta G'$
		mM	Kcal/mol
Matrix	3.9	3.5	-12·9
Extramitochondrial	29	0.48	-15.2

The ATP/ADP ratio was found to be approximately 7 times as high in the suspending medium as in the matrix. Using this ratio and the measured Pi concentrations it is possible to calculate the free energy of hydrolysis of ATP, i.e. the phosphorylation potential, for both compartments. The phosphorylation potential found for ATP in the medium is 2·3 Kcal/mol more negative than for the ATP in the matrix.

Similar differences between the ATP/ADP ratios in the cytosol and mitochondria in the *in vivo* situation have been reported in preliminary experiments by Elbers *et al* using perfused rat liver (64). This group have devised a technique using freeze-drying, homogenisation in heptane and separation of cell components by heptane-carbon tetrachloride gradients to measure adenine nucleotide concentrations in the above tissue after quenching in liquid nitrogen. These results presumably would lead to similar phosphorylation potentials to those calculated above.

Inhibition of the translocation of ATP in the control state is linked to the increase in the phosphorylation potential of the exogenous ATP. Energy involvement in creating the potential difference between the exogenous and endogenous adenine nucleotide systems has been shown using uncouplers. In the uncoupled state the ATP/ADP ratios in the matrix and extramitochondrial spaces approach one another (97,122). This equilibration is a direct consequence of the breakdown of the membrane potential by the uncoupler which abolishes the specificity of the translocase for ADP (see above).

It has been proposed that the energy to produce this translocation specificity and consequently the phosphorylation potential difference, comes from electron transport in the form of H^+ ions. According to Klingenberg *et al* (122) only a small portion of the H^+ generated by electron transport need 'leak back' in order to allow an increase in the phosphorylation potential.

A direct consequence of this mechanism for generation of phosphorylation potential is that the theoretical P/O ratios of 3 for substrates that enter the respiratory chain at NADH, and 2 for substrates that enter at the flavoprotein such as succinate

are never attained (115). The physiological importance of these observations is twofold. Firstly the addition of translocation energy to phosphorylation energy is an efficient means of increasing the phosphorylation potential above that primarily generated during phosphorylation. Secondly it has been suggested that the cell may have a preference for a certain number of energy equivalents of high potential instead of a larger number of energy equivalents at lower potential (122). In terms of translocation of adenine nucleotide the translocation energy is used to transport ADP into and ATP out of the cell against a concentration gradient.

Evidence to support the above has been reported by Kalstein and Klingenberg (115) who found that the P/O ratio is inversely proportional to the external ATP potential or ATP/ADP ratio. When the ATP/ADP ratio was kept artificially low by the use of a glucose hexokinase trap the P/O ratios were found to approach the theoretical values.

An *in vivo* situation where one would logically expect the extramitochondrial ATP/ADP ratio to be low is in the heart. Jacobus and Lehninger (111) have shown that the presence of the enzyme creatine kinase in the intermembrane space results in the almost immediate dephosphorylation of ATP produced by oxidative phosphorylation to form creatine phosphate. Under these circumstances and with a plentiful supply of creatine as acceptor one would expect the steady state concentration of extramitochondrial ADP to be relatively high. Thus the cytoplasmic phosphorylation potential would fall and as a result of less translocation energy being required, P/O ratios would approach 2 or 3 as the case may be.

Energy charge of the cell

As an index of the energy level of the cell Atkinson and Walton (2,4) have introduced a line of thinking relative to the 'energy charge' of the cell. This is defined as half of the number of anhydride-bound phosphate groups per adenosine or

$$\left(\tfrac{1}{2}\,\frac{2ATP + ADP}{ATP+ADP+AMP}\right).$$

Experimental systems have been utilised to

show that the activities of a number, at least, of adenine nucleotide metabolising enzymes are dependent not so much on the absolute concentrations of the adenine nucleotide but rather depend entirely on the ratio of their concentrations (the energy charge).

Cellular concentrations of ATP tend to remain constant. The utilisation of endogenous ATP can be offset to some extent by reserves of ATP in the form of ADP which is converted to ATP and AMP by adenylate kinase and creatine phosphate which can be dephosphorylated to form ATP and creatine. Thus these systems may be said to act as a short term ATP 'buffer'.

These reactions also play a very important role in the adenine nucleotide metabolism of the cell by way of providing prior warning that ATP is becoming limiting. This is well illustrated by the effect of AMP formed by the adenylate kinase reaction on the activity of phosphofructokinase a control point enzyme in glycolysis (193). AMP is an allosteric effector of this enzyme and by inducing a change from sigmoid to Michaelis-Menton behaviour stimulates the glycolytic pathway resulting ultimately in the production of reducing equivalents which are oxidized in the mitochondrion concomitantly with the formation of ATP.

A similar situation applies in muscle where, after stimulation, a pronounced increase in respiration occurs before the creatine phosphate stores are exhausted (58).

Because of the energy-dependent preference for the transport of ADP into the mitochondrion, a small increase in the ADP concentration in the extramitochondrial space leads to a marked increase in the ADP concentration in the matrix space, with the result that phosphorylation in the mitochondrion is stimulated. The importance of the role of adenine nucleotide translocase in this stimulation has been demonstrated by Heldt *et al* (97). On the basis of a ADP/ATP translocation rate of 10 (see above) they have calculated that the half-maximum rate of ATP synthesis would be reached if only 9% of the external adenine nucleotide is present as ADP. Under these conditions the ATP/ADP ratio in the matrix is unity (96,97).

Redox Potentials

Adenine nucleotide phosphorylation potentials are linked to the redox potentials of the pyridine nucleotide couples by enzymes which establish equilibrium (132,269). These links have been hypothesised to involve both mitochondrial and cytoplasmic couples. In this way the basic level of the redox states of the two pyridine nucleotide couples in the two main cell compartments are intimately associated with the ATP level in the cell. A full discussion of the myriad of interrelationships and reactions involved in these equilibria is outside the scope of this introduction. It suffices to say that variations, either in the phosphorylation potential or redox potentials, leads to predictable changes in the other connected systems which in turn leads to metabolic changes in the cell in an effort to restore the *status quo*.

Large differences are observed in redox potentials in both cellular compartments and between $NAD/NADH_2$ and $NADP/NADPH_2$ couples (132,269). This reflects the different degrees of reducing and oxidizing power required for the functions carried out in a particular cell compartment by a particular pyridine nucleotide. For example in the cytoplasm the $NAD/NADH_2$ ratio is high (NAD acts as an oxidizing agent in glycolysis) whilst the $NADP/NADPH_2$ ratio is low ($NADPH_2$ acts as a reducing agent in biosynthetic reactions).

Objectives of Thesis Research

Being a membrane-bound enzyme, the functioning of adenine nucleotide translocase must be intimately involved with the overall structure of the inner mitochondrial membrane. As such the activity of the translocase, a transporter of charged molecules, would be influenced by charged agents which are capable of binding to this membrane. Preliminary experiments indicated that Ca^{2+} was one such agent in that it stimulated the translocation of ATP (234). In the light of these points the studies reported in this thesis are aimed at the further characterisation of the adenine nucleotide translocase in rat liver mitochondria with particular importance being placed on the mechanism by which Ca^{2+} stimulates this process. Central to this theme has been an investigation into the effect of perturbators

of the protein-phospholipid interactions, with emphasis being placed on the involvement of phospholipids, on the translocase reaction.

In section C of this thesis the kinetic properties of the Ca^{2+}-stimulated adenine nucleotide translocation have been determined.

Section D outlines the effect of membrane-active agents, such as local anaesthetics, both on the translocase and the interactions with Ca^{2+}.

In section E the involvement of phospholipids in the action of the translocase is carried a step further by means of hydrolysing specific mitochondrial phospholipids and monitoring a a variety of parameters associated with adenine nucleotide translocation therein.

SECTION B EXPERIMENTAL

Materials

[^{14}C]ATP, [^{3}H]ATP, [^{14}C]ADP, [^{3}H]ADP, [^{14}C]Sucrose, $^{3}H_2O$ and $^{45}CaCl_2$ were obtained from The Radiochemical Centre,Amersham, Bucks., England. Carrier-free [^{32}P]phosphate was obtained from The Australian Atomic Energy Commission, Lucas Heights, Sydney, NSW, Australia. Hexokinase (EC 2.7.1.1), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), pyruvate kinase (EC 2.7.1.40), adenylate kinase (EC 2.7.4.3), lactate dehydrogenase (EC 1.1.1.27), potassium carboxyatractylate (carboxyatractyloside or gummiferin) and all nucleotides were purchased from C.F. Boehringer und Soehne, G.m.b.H., Mannheim, West Germany. Other chemicals were obtained as follows: Rotenone, antimycin A, oligomycin, gramicidin D, bovine serum albumen (fraction V), HEPES, lyophilised *Crotalus adamanteus* (eastern diamondback rattlesnake) venom, CCCP, oleic acid, linoleic acid, arachidonic acid, MES and DEAE-cellulose from Sigma Chemical Co., St. Louis, Mo., USA; potassium atractylate (atractyloside) and valinomycin from Calbiochem, Los Angeles, California, USA; Soluene and Butyl-PBD from Packard Instrument Co., Downers Grove, Ill., USA; $CaCl_2$ as a 0.1M standard solution from Orion Research Inc., Cambridge, Mass., USA; $LaCl_3.7H_2O$ from Ajax Chemicals Ltd., Sydney, NSW, Australia; $Nd(NO_3)_3$ from Hopkin and Williams Ltd., Chadwell Heath, Essex, England; Ruthenium Red from Schmid and Co., Stuttgart-Untertürkheim, Germany; $U(NO_3)_2$ from May and Baker Ltd., Dagenham, England; DEAE-cellulose paper (Whatman DE 81), silica gel loaded paper (Whatman SG 81) and CM-cellulose (Whatman CM 11) from W & R Balstron Ltd., England; Tetracaine from Glaxo Australia Pty. Ltd., Boronia, Victoria, Australia; Nupercaine-HCl from CIBA Pharmaceuticals, Crows Nest, NSW, Australia; Butacaine sulphate from Abbott Australia Pty. Ltd; Procaine-HCl from Drug Houses of Australia Pty. Ltd.; Lysophosphatidylcholine and lysophosphatidylethanolamine from Koch-Light Laboratories Inc., Colnbrook, Bucks., England; Sephadex G-50 and G-100 from Pharmacia Fine Chemicals AB, Uppsula, Sweden; 2,6-Di-tert-butyl-p-cresol (butylated hydroxytoluene) from British Drug Houses Ltd., Poole, England; Bio-Rex 70(100-200 mesh) from Bio-Rad Laboratories, Richmond, California, USA. Fresh pig pancreas was obtained from the city slaughterhouse.

Methods

Isolation of mitochondria

Wistar strain albino rats of approximately 200g body weight were used in all experiments. They were maintained on a standard rat meal diet and starved overnight before use to lower the glycogen content of the preparations. Animals were stunned by a blow on the head and killed by decapitation. The livers were rapidly removed and placed in ice-cold homogenisation medium containing 250mM sucrose, 2mM HEPES and 0.5mM EGTA buffer adjusted to pH 7·4 with KOH. The liver was then minced with scissors and homogenised with a glass-Teflon tissue disintegrator (Arthur H. Thomas Co., Philadelphia, Pa., USA; size C) by one up and down stroke aided by a motor driven at 900 rev./min. The resulting suspension was made up to 90 ml with medium and the mitochondria prepared essentially by the method of Meyers and Slater (171). This comprised an initial low speed spin at 2500 rev./min (600g) for 5 min in a Sorvall RC-2B refrigerated centrifuge (Ivan Sorvall Inc., Newtown, Conn., USA.) fitted with the SS-34 rotor. The supernatant from this centrifugation was retained and the pellet rehomogenised in the glass-Teflon tissue grinder by means of 10 up and down strokes performed by hand in homogenisation medium and recentrifuged as before. The combined supernatants were then centrifuged three times at 11,000rev./min. for 5 min. Washes were performed using a medium as described above except that the EGTA was omitted and the final mitochondrial pellet resuspended in this medium at a concentration of 40mg of mitochondrial protein/ml.

Preparation of mitochondria for 'back-exchange' experiments

(For theory on the use of the back-exchange technique see ref.199)

Samples of the mitochondrial suspension, containing 100mg of protein, were incubated with 2μCi of [^{14}C]ATP or [^{14}C]ADP or with 5μCi of [^{3}H]ATP or [^{3}H]ADP for either 30 min at 4°C or 1hr at 0°C to homogenously label the endogenous adenine nucleotide pool. These mitochondria were then centrifuged (16,000g for 5 min in the Sorvall centrifuge) and washed twice by centrifugation in EGTA-free isolation medium and finally resuspended to the original protein concentration.

Incubation conditions

All experiments were performed in a medium consisting of 200mM sucrose containing 2mM HEPES-KOH (pH 7·4) and at a temperature of 4°C (except for Arrhenius determinations) with 1-2mg of mitochondrial protein/ml present. Other additions and conditions were as indicated in the legends to figures and tables.

Incubation methods

Three different types of incubation system were used. (a) Time-course determinations, using the 'back-exchange' technique were done in a thermostatically-controlled water-jacketed glass incubation vessel (vol. 1.5ml). Stirring was accomplished by means of a small Teflon-coated magnet. Mitochondria were introduced to the reaction medium 1min before starting the reaction unless otherwise indicated. The reaction to be studied was initiated by the addition of non-radioactive adenine nucleotide with or without Ca^{2+} as indicated. At predetermined time-intervals, portions of the incubation mixture were removed, added to an Eppendorf Microfuge tube containing 50μM atractyloside and the tube contents mixed on a Vortex-Genie mixer (Scientific Industries Inc., Springfield, Mass., USA.). Approximately 10s before initiating the reaction, a sample was removed to determine the unspecific leakage of endogenous labelled adenine nucleotide and added to an Eppendorf Microfuge tube containing 50μM atractyloside plus adenine nucleotide (and Ca^{2+}). This sample was used in calculating the zero-time control. The shortest reaction time obtainable by this method was 5s.

(b) Determination of the initial rates of reactions were performed in Eppendorf Microfuge tubes. The reaction was initiated by the addition of a portion of the mitochondrial suspension, unless otherwise indicated. The final volume was 250μl. Reactions were stopped, usually after 10 or 20s, by the addition of 50μM atractyloside. Mixing, both on the addition of mitochondria and of atractyloside, was performed by gentle agitation on a Vortex-Genie mixer. The tubes were temperature-equilibrated by insertion in a specially designed Perspex rack placed in a water bath. In both of these methods the mitochondria were separated from the incubation mixture by centrifugation in an Eppendorf bench centrifuge for 2min. Methods (a) and (b) were used only for back-exchange reactions. Control experiments indicated that both methods gave quantitatively similar results.

(c) Forward-exchange experiments were performed as in (a) or (b) above with [^{14}C]ATP or [^{14}C]ADP. The reaction was stopped in the usual way with atractyloside and the supernatant removed by aspiration with a water-driven vacuum pump. In dual-labelling experiments (see Section C) a sample of this supernatant was retained and counted for radioactivity. The mitochondrial pellet was then washed with 0·5ml of the basic incubation medium and recentrifuged. The supernatant was again removed and the pellet dissolved in 0·1ml of Soluene by heating in an oven at 60°C for 1hr.

Ca^{2+} accumulation by mitochondria

This was measured using the method of Reed and Bygrave (209). Essentially this involves the addition of 1mM EGTA to the mitochondrial suspension which not only chelates the free Ca^{2+}, thus terminating the accumulation process, but also removes Ca^{2+} bound at the large number of mitochondrial low affinity binding sites. A 100µl sample of the supernatant obtained after sedimenting the mitochondria was removed and counted to determine the degree of accumulation. Using this technique only the Ca^{2+} ions which are actually transported across the inner membrane of the mitochondria are measured.

Phospholipase treatment of mitochondria

This was performed at 25°C in a medium of the following composition: 200mM sucrose, 20mM HEPES-KOH pH 7·4, 1mM $CaCl_2$, 1% defatted BSA and mitochondrial protein at 10mg per ml. After a one minute preincubation period phospholipase was added at a concentration of 20µg per mg of mitochondrial protein and, at the required times, the reaction terminated by the addition of 10 volumes of ice-cold medium containing 200mM sucrose, 2mM HEPES-KOH pH 7·4, 0·5mM EGTA and 0·5% defatted BSA. This suspension was left on ice for 5 minutes and the mitochondria then sedimented by centrifuging for 10 minutes at 14,000g in a Sorvall centrifuge. The pellet was resuspended after one wash in the above medium, but without BSA and EGTA, at a protein concentration of 14mg per ml (calculated on the basis of original protein added).

Preparation of (^{32}P)-labelled mitochondrial membranes

200gm male wistar rats were injected intraperitoneally with 0·5mC carrier-free [^{32}P]phosphate. 40 hours later the animals were sacrificed, mitochondria isolated and phospholipase digestions performed as indicated above in a total volume of 8·0 ml. Aliquots (0·5ml) were removed at the desired times and the reaction stopped by the addition of 2mM EGTA (final concentration).

Extraction of (^{32}P)-labelled phospholipid from mitochondrial membranes

Phospholipid extractions were performed essentially by the method of Dawson *et al* (52) 4ml of chloroform: methanol (2:1) was added to the mitochondrial sample described above and homogenised at room temperature in a Thomas type A glass-teflon tissue grinder using a motor-driven pestle. The organic phase was removed using a pasteur pipette and filtered through a glass-fibre disc under vacuum into a stoppered test-tube. The residue was extracted twice more using chloroform:methanol (7:1) saturated with ammonia and finally with chloroform:methanol (2:1). After the final extraction both the aqueous and organic phases were filtered. 3ml of 0·9% NaCl was added to the filtrate, the tube mixed vigorously and the Folch-type partition (74) performed overnight at 2°C. The upper aqueous phase was then discarded and the lower phase dried over Na_2SO_4 before being evaporated to dryness in a Buchi rotary evaporator under vacuum at room temperature. The lipid residue was taken up in 200µl of chloroform:methanol (2:1) and an aliquot chromatographed on thin layer silica-gel paper.

Separation of phospholipids by thin layer chromatography

The lipid extract (50µl) was applied to one corner of a 20cmx20cm sheet of silica-gel impregnated filter paper in a 1cm diameter spot. Individual phospholipids and their lyso derivatives were then separated on this chromatogram using a two-dimensional system consisting of chloroform:methanol: 28% aqueous ammonia (65:35:5) and then, after evaporation of the solvent for ½ hour in a fume-hood, chloroform:acetone:methanol:glacial acetic acid: water (10:4:2:2:1)(67). Phospholipids were detected using a solution of 0·0012% Rhodamine and viewing the treated chromatogram under UV light, identified using known R_f values and standards, cut out and counted in a Scintillation spectrophotometer.

Depletion of endogenous adenine nucleotides from mitochondria

Depletion was carried out essentially according to the method of Erdelt *et al* (66). This involved incubating rat liver mitochondria at a concentration of 0·25mg protein per ml in a medium consisting of 50mM phosphate buffer, 50mM sucrose and 5mM $MgCl_2$ (pH 7·4) for 15 minutes at 25°C. The suspension was centrifuged at 15,000g for 10 minutes, washed with 250mM sucrose, 10mM HEPES-KOH, 2mM EDTA, pH 7·0 and recentrifuged. The final resuspension was in 250mM sucrose, 5mM HEPES-KOH, pH 7·0 at a concentration of 40mg protein per ml (calculated on the basis of original protein added).

Binding of adenine nucleotides

Binding of ATP to the mitochondrial membranes was assayed by the method of Weidemann *et al* (267). Mitochondria were suspended in Eppendorf tubes at 2mg protein per ml in 0·5 ml of medium containing 250mM sucrose, 5mM HEPES, 5mM MES, 2mM EDTA and 1mM Ado-3'-P (pH'ed to 7·0 with KOH). At each ATP concentration 3 samples were prepared: (a) no atractyloside added and the reaction terminated by centrifugation of the mitochondrial suspension after 2 minutes; this sample represents specific binding plus non-specific binding plus a small amount of residual translocation activity, (b) 50µM atractyloside added before ATP; this sample represents only non-specific binding, and (c) 50µM atractyloside added after 2 minutes of incubation with ATP; this sample gives non-specific binding plus translocation activity. Specific binding to the translocase molecule is represented by the difference between (a) and (c). After a 2 minute incubation the samples were centrifuged in an Eppendorf Microfuge for 1 minute, the supernatant aspirated off using a water-driven vacuum pump, 1ml of wash medium containing 250mM sucrose, 5mM HEPES, 5mM MES plus 2mM EDTA (pH 7·0) added and the centrifugation/aspiration step repeated. Soluene (100µl) was then added, the tubes homogenised by vibration against a rubber cross-bar mounted on an electric motor and placed in an incubator at 60°C for 1 hr to digest the protein. After digestion the samples were quantitatively transferred to vials containing 10ml of scintillation fluid and counted.

Analytical methods

Mitochondrial protein was determined using a modified biuret method which corrected for turbidity of the samples (243). This was achieved by the addition of a small amount of KCN to the cuvettes, after the initial reading had been taken, which formed a colourless complex with the copper reagent. The OD readings were then taken once more and the residual OD, due to turbidity of the sample, subtracted from the original to give the correct reading. The readings after KCN decolorisation were of the order of 10-15% of the original. ATP was assayed by the method of Lamprecht and Trautschold (136) and ADP and AMP by the method of Adam (1). Radioactive adenine nucleotides were analysed for radiochemical purity by chromatography on DEAE-cellulose paper according to Morrison (180). Carrier amounts of adenine nucleotide were added in the form of 5µl of 10mM solutions. Cytochrome a contents were measured, following reduction of the test cuvette with dithionite and oxidation of the reference cuvette with ferricyanide, with a split beam Varian-Techtron model 635 digital readout spectrophotometer. Results were quantified from the ΔA (605-630nm) using a ΔE(reduced-oxidized) of $18 \cdot 0 cm^{-1} mM^{-1}$.

Oxygen consumption

Mitochondria were regularly checked for their intactness, using the respiratory control ratio as an index (37), by measuring oxygen consumption in a medium of the following composition: 100mM sucrose, 50mM KCl, 15mM HEPES, 10mM Pi, 2mM $MgCl_2$, 1mM EDTA and 10mM succinate, pH 7·4 (see ref.208). The amounts of inhibitors to be used in the section on energy requirements were tested using the above system.

Radioactivity measurements

Radioactivity was determined by counting a 100µl sample of the supernatant (back-exchange experiments) or the solubilised mitochondrial pellet (forward-exchange experiments). The samples were dissolved in 10ml of scintillation fluid (6g of Butyl PBD, 400ml of methoxyethanol and 600ml of toluene) and counted to 1% error either in a Packard Tri-Carb or Beckman LS-100 scintillation spectrophotometer on appropriate channel settings. All c.p.m. were converted into d.p.m. using an external standard.

Purification of phospholipase A

Phospholipase A has been prepared to a high degree of purity from a variety of sources including brain (78,79), pancreas (55, 160), bee venom (228), macrophages (77), heart (76), bacteria (59) and snake venom (5,49,233,268,277). The two phospholipases that have been utilised in the experiments described in Section E were those from porcine pancreas (55) which hydrolyses phospholipids in the order cardiolipin >phosphoatidylethanolamine > phosphatidylcholine and from *Crotalus adamanteus* venom (268) which hydrolyses phospholipids in the order phosphoatidylcholine > phosphatidylethanolamine with cardiolipin being inactive as a substrate.

Pancreatic phospholipase

The final method for purification of a large amount of this enzyme was essentially as described by de Haas *et al* (55) and was as follows: Fatty deposits were removed from a sample of fresh porcine pancreas (obtained from approximately 5 animals). 350gm of this material was homogenised for 3 minutes at top speed in a Waring blendor with 1 litre of 0·15M NaCl. 2ml of toluene was added to inhibit bacterial growth and the homogenate stored overnight at room temperature. This step was essential to obtain maximal activity as the phospholipase exists *in vivo* as a prophospholipase (56). Conversion to the active phospholipase requires the cleaving off of a small polypeptide by endogenous protease activity during this incubation period. After the incubation period the homogenate was brought to pH 4·0 with conc. HCl and heated at 70°C for 3 minutes in a water bath. Low pH was necessary to dissociate the lipid-enzyme complex derived from the homogenate and solubilise the phospholipase. Heat treatment resulted in the denaturation of a large proportion of the protein and a consequent increase in the specific activity. Due to its amino acid composition, pancreatic phospholipase A is a very stable molecule and is unaffected both by the low pH and high temperature. Denatured protein remaining after the above treatment was removed by centrifugation at 4000g for 30 mintes in a Sorvall RC-2B centrifuge after the solution had been rapidly cooled to 0°C. The remaining floating fat layer was filtered using first Whatman filter paper and secondly a millipore filter of 0·45μ pore size. The resulting yellow solution was dialysed for 12 hours against running tap water, cleared by centrifugation at 10,000g for

30 minutes in a Sorvall centrifuge and subjected to fractional precipitation with solid $(NH_4)_2SO_4$ at pH 7·0 and O°C. A 0.40 to 0·60 saturationof $(NH_4)_2SO_4$ cut was taken and the resulting precipitate dissolved in 5ml of 0·75M NaCl and the turbid solution cleared by centrifugation at 45,000g for 15 minutes. The supernatant was then chromatographed at 4°C on Sephadex G-50 using 0·75M NaCl as eluent (column dimensions, 90cm x 2·5cm; sample volume, 5ml; flow rate, 100ml per hour). Contrary to the elution pattern obtained by de Haas *et al* (55) the majority of the phospholipase activity was found in the void volume peak. This was ascribed to asorption of the enzyme to lipid droplets as described above and coroborated by the observation that there was a time lag in the attaining of maximal activity in the assay system and that this time lag could be abolished by preincubating with detergent. To remove the lipid contaminant the active fractions were extracted 5 times with isobutanol/butanol (3:7). The last extraction was performed in the cold-room for 2 weeks after which the resulting aqueous phase was dialysed against 0·75M NaCl, concentrated using Diaflow apparatus (Model 402) with a PM-10 membrane and reapplied to the Sephadex G-50 column as above. On this occasion the enzyme peak was obtained approximately 20 fractions after the void volume. Active fractions were pooled and dialysed against distilled water and then 5mM Tris buffer (pH 8·0) for 8 hours. This solution was cleared by centrifugation at 45,000g for 15 minutes in a Sorvall centrifuge, concentrated to 10ml as above and chromatographed on a DEAE-cellulose column (column dimensions, 50cm x 2.5cm; sample volume, 15ml; flow rate, 100ml per hour) at 4°C equilibrated against 5mM Tris buffer (pH 8·0). After application of the sample 20 fractions were collected using the Tris buffer as eluent and the remaining fractions eluted with a linear gradient using 0·3M NaCl in 5mM Tris (pH 8·0). Active fractions were collected, dialysed against two changes of 5mM acetate buffer (pH 6·0) and concentrated to 10ml. Rechromatography was performed on CM-cellulose equilibrated against the same buffer (column dimensions, 50cm x 2·5cm; sample volume, 5ml; flow rate, 100ml per hour) and the enzyme eluted with a linear gradient using 0·4M NaCl. Active fractions were combined, dialysed against distilled water, lyophilised and stored at -20°C.

Crotalus adamanteus venom phospholipase

1gm of lyophilised venom was suspended in 10ml of 0·1M NaCl, 0·05M Tris and 1mM EDTA (pH 8·0). After mixing for 5 minutes the solution was centrifuged at 6000g for 5 minutes and the clear yellow solution chromatographed on Sephadex G-100 (column dimensions, 90cm x 2·5cm; sample volume 5ml; flow rate, 25ml per hour). Active fractions were collected, dialysed against 0·15M Na phosphate buffer (pH 6·8) and concentrated to 10ml using Diaflow apparatus and a PM-10 membrane. This solution was then chromatographed on Bio-Rex 70 (100-200 mesh), a weak cation exchange resin, using 0·15M Na phosphate (pH 6·8) as an eluent (column dimensions, 45cm x 2·5cm; sample volume, 5ml; flow rate, 15ml per hour). After dialysis of the active portions against distilled water and several changes of 0·05M Tris buffer (pH 8.0) and concentrating as above, the solution was rechromatographed on DEAE-cellulose (column dimensions, 45cm x 2·5cm; sample volume, 5ml; flow rate, 100ml per hour). After elution with a linear gradient formed from 0.05M Tris (pH 8.0) and 0·2M NaCl two active peaks were obtained. The fractions corresponding to both of these active peaks were collected, dialysed against distilled water, lyophilised and stored at -20°C.

Stock solutions of both the pancreatic and venom phospholipases were prepared on the day of use in distilled water to a concentration of 5mg of protein per ml.

Phospholipase activity assay

During the purification procedures the phospholipase activity of various column fractions was assayed in the following manner (see ref.55): One egg yolk was homogenised in 100ml of water and diluted one to three in a solution containing sodium deoxycholate and $CaCl_2$ at final concentrations of 0.2% and 5mM, respectively. 2ml of this suspension at pH 8·0 was added to a glass water-jacketed incubation vessel at 40°C. Fatty acids enzymically released from the egg phosphatidylcholine were assayed by following the decrease in pH using a Townson and Mercer expanding pH meter linked to a Rikadenki chart recorder after the addition of appropriate amounts of the phospholipase enzyme fraction. The system was calibrated by back-titrating with a standard KOH solution.

Preparation of samples for electron microscopy

Mitochondria were incubated for varying times with phospholipase A and reactions stopped in the usual way (see above). Mitochondrial pellets (containing 4mg protein) were sedimented in 15ml Corex centrifuge tubes at g values varying between 8,000 for 10 minutes (controls) to 40,000 for 15 minutes. These pellets were then treated with a solution of 3% glutaraldehyde in 200mM sucrose, 40mM HEPES-KOH pH 7·4 at 0°C for 3 hours. The resulting fixed samples were washed overnight with the above buffer and then post-fixed with a 1% osmium tetroxide/buffer and again washed. After block staining in 4% aqueous uranyl acetate and being washed briefly with buffer the samples were dehydrated in a series of alcohol/water mixtures of increasing concentration for 5 minutes each and then twice for the same time using absolute ethanol. Alcohol was removed by two 5-minute washes in propylene oxide and then treated for ½ hour with propylene oxide/Spurrs embedding medium (3:1) followed by propylene oxide/Spurrs (1:3) for ½ hour. Finally the samples were washed four times with Spurrs, twice for 5 minutes, once for 2 hours and lastly overnight. Embedding was performed at 70°C for 12 hours. Silver-gold sections were cut on a Reichert OM U2 ultramicrotome using glass knives, collected on 200 mesh copper grids coated with pallodion, post-stained with 4% uranyl acetate for 20 minutes followed by Sato's lead stain for 3 minutes and viewed in a Hitachi HU 12 electron microscope.

Calculations

Translocation data were analysed essentially as indicated by Pfaff and Klingenberg (199) and Pfaff *et al* (201). To do the analyses, the size of the readily exchangeable endogenous adenine nucleotide pool present in the mitochondria had to be determined. In the case of liver mitochondria this pool was approximately equal to the content of ATP plus ADP. This is not the case in heart (267) and Krebs ascites tumour cell mitochondria (247) where the exchangeable pool is much less than the sum of ATP and ADP.

Determination of % exchange of endogenous adenine nucleotide

This is defined in the case of 'forward-exchange' type experiments as being equivalent to $\frac{\text{sp. act.}_{in}}{\text{sp. act.}_{ex}} \times \frac{100}{1}$

where sp. act.$_{in}$ = specific activity of endogenous adenine nucleotide and sp. act$_{ex}$= specific activity of exogenous adenine nucleotide.

(a) $$\text{sp. act.}_{in} = \frac{\text{c.p.m.}_{in}}{\text{nmoles (ATP+ADP)}_{in}}$$

where c.p.m.$_{in}$ = c.p.m.(test pellet) - c.p.m. (zero time control pellet).

(b) $$\text{sp. act.}_{ex} = \frac{\text{c.p.m.}_{ex}}{\text{nmoles adenine nucleotide}_{ex}}$$

where c.p.m.$_{ex}$ = total c.p.m. added - c.p.m.$_{in}$ and

nmoles$_{ex}$ = nmoles of radioactive ATP or ADP added.

Usually c.p.m.$_{ex}$ = total c.m.p. added.

Using the 'back-exchange' technique the % exchange is equivalent to $\frac{\text{c.p.m.}_{ex}}{\text{c.p.m.}_{in}} \times \frac{100}{1}$

where c.p.m.$_{ex}$ = c.p.m. (test supernatant) - (zero time control supernatant)

and c.p.m.$_{in}$ = total exchangeable c.p.m. in the mitochondria - c.p.m. (zero time control supernatant)

Total exchangeable c.p.m. is a value corrected for the % of non-exchangeable AMP. Endogenous adenine nucleotides are homogeneously labelled during the incubation of the mitochondria with radioactive ATP or ADP. Thus it is not surprising to find that the % of radioactivity contained in the AMP portion was approximately equal to the % of AMP in total adenine nucleotide. Usually 70 ± 5% of the radioactive label is contained in ATP plus ADP.

Determination of absolute rates of translocation

Once the % exchange has been determined and knowing the endogenous content of (ATP + ADP) it is possible to calculate the translocation activity in terms of nmoles translocation/min per mg

of mitochondrial protein. This rate is determined using first-order kinetics and is equivalent to

$$2.303.\text{nmoles (ATP + ADP)}.\ 1/\text{time(min)}.\log\left(\frac{100}{100-\%\text{ex}}\right)$$

The majority of experiments described in this thesis were performed using the 'back-exchange' instead of 'forward-exchange' method for the following reasons: (a) it is much quicker; one only has to remove a 100µl sample for radioassay, (b) zero time controls give an indication of the intactness of the membrane, (c) one may easily detect changes in the composition of the endogenous pool by chromatography of a small extract, and (d) it was found to give much more accurate and reproducibe results.

Quantification of binding data

Scatchard plots (216) were used to obtain approximate binding constants from the binding data. Using these values as a starting point and with the facilities provided by a PDP 8/I computer and a Hewlett Packard graph plotter the 'correct' binding constants were determined using a trial and error basis.

Kinetic constants were determined by the use of double-reciprocal plots. In the case of activators or inhibitors the reciprocal of the % activation or inhibition was plotted against the reciprocal of the activator or inhibitor concentration.

The concentrations of 'free' Ca^{2+} and 'free' ATP were calculated from the known stability constants of the $CaATP^{2-}$ and $CaHATP^{-}$ species (231,245). Similar constants were also used to calculate 'free' metal concentrations in other systems.

SECTION C: METAL IONS AND ADENINE NUCLEOTIDE TRANSLOCATION

Introduction

During investigations carried out on the ATP-dependent accumulation of Ca^{2+} by rat liver mitochondria, it became apparent that the translocation of ATP was stimulated in the presence of Ca^{2+} ions (236). This stimulation differed from that described by Carafoli *et al* (31,146) in that it was insensitive to DNP. These workers had shown that in the presence of high (4mM) Ca^{2+} concentrations, mitochondria were able to bind up to 160nmoles ATP per mg protein. These results are difficult to explain on the basis of a 'one-for-one' exchange of adenine nucleotide via the translocase as this concentration of adenine nucleotide is about 10 times of that in freshly isolated mitochondria (267). They do however provide the first direct experimental evidence that adenine nucleotide translocation may be influenced by the ions present in the suspending medium.

In some initial studies on the translocase Pfaff and Klingenberg (199) found that Mg^{2+} ions slightly inhibited ATP translocation and slightly stimulated ADP translocation. Later results reported by Pfaff *et al* (201) indicated that 4 mM Mg^{2+} inhibited both the extent of and the affinity for the translocation of ATP and ADP. Similar responses were observed by Duée and Vignais with respect to ADP translocation (they did not test ATP) (62). Inhibition of ADP translocation is not difficult to explain on the basis of a conversion to ATP and AMP by adenylate kinase in the intermembrane space. AMP is not translocated whilst ATP is translocated at a rate significantly less than that for ADP. By analogy one would expect added ATP to be partially converted to ADP which would stimulate the translocation rate; this increase was not observed (199).

Monovalent cations, such as K^{+} have been found to stimulate both ATP and ADP translocation, the former to a greater extent than the latter (199).

Against this background it was decided to investigate the Ca^{2+} mediated stimulation of adenine nucleotide translocation. This section describes results obtained from such a study. The

approach was as follows: initially the system was characterised as regards bivalent metal ion and adenine nucleotide specificities and the involvement of energy. Then various parameters such as the effects of the simultaneous addition of K^+ and Mg^{2+} ions to the Ca^{2+}-stimulated system, the effect of Ca^{2+} accumulated in the mitochondrion and the antagonism by trivalent rare earth cations were investigated. The involvement of membrane charge in the phenomenon was also tested by determining the effect of cations on atractyloside inhibition of the translocation process.

Some consequences of the Ca^{2+} stimulation with respect to the metabolism of the cell are discussed and a scheme proposed whereby Ca^{2+} accumulation and ATP translocation are 'linked'. Evidence for a proposed mechanism of action of Ca^{2+} in stimulating adenine nucleotide translocation is also presented and tabulated.

Results

Figure 1 shows the influence of 200μM added Ca^{2+} on the time-course of exchange of an equimolar concentration of ATP by isolated rat liver mitochondria measured by the back-exchange technique. The rate of exchange was stimulated approximately two-fold in the presence of the added Ca^{2+}. This is more easily seen from the data shown in Figure 2 where the data from Figure 1 was recalculated and expressed in terms of a first-order kinetic plot. First-order constants derived from this plot are 0·43 min^{-1} in the absence of 0·84 min^{-1} in the presence of Ca^{2+}. Zero order rate constants may be obtained from these values by multiplying by the content of endogenous exchangeable adenine nucleotide (in this case 11nmol per mg protein). These are 4·7 and 9·2nmol/min per mg protein respectively.

Other experiments have shown that measurements involving the forward-exchange technique give quantitatively similar results to those obtained with the back-exchange technique (see for example, Table VII). The leakage of labelled adenine nucleotide from the mitochondria remained low and approximately constant for up to 15 min in the absence and presence of Ca^{2+}. These findings indicate that Ca^{2+} was causing little membrane rupture during the period of incubation.

Zero-time values for the above experiment were 4·8% and 7·0% in the absence and presence of 200μM Ca^{2+} respectively. Approximately 2·6% of both these values was contributed to by labelled adenine nucleotide which had either leaked out of the mitochondria prior to the start of the experiment or had not been washed from the suspending medium after the labelling procedure. Thus the corrected zero-time values of 2·2% and 4·4% are directly proportional to the activity of the translocase in each case (see above). This is due to the fact that atractyloside, being a competitive inhibitor, does not produce complete inhibition unless added at very high concentrations.

Effect of Ca^{2+} concentration on the translocation of ATP

Data in Figure 3 show the effect of increasing the added Ca^{2+} concentrations on the exchange (hereafter referred to as translocation) of 200μM ATP. The slight deviation from the

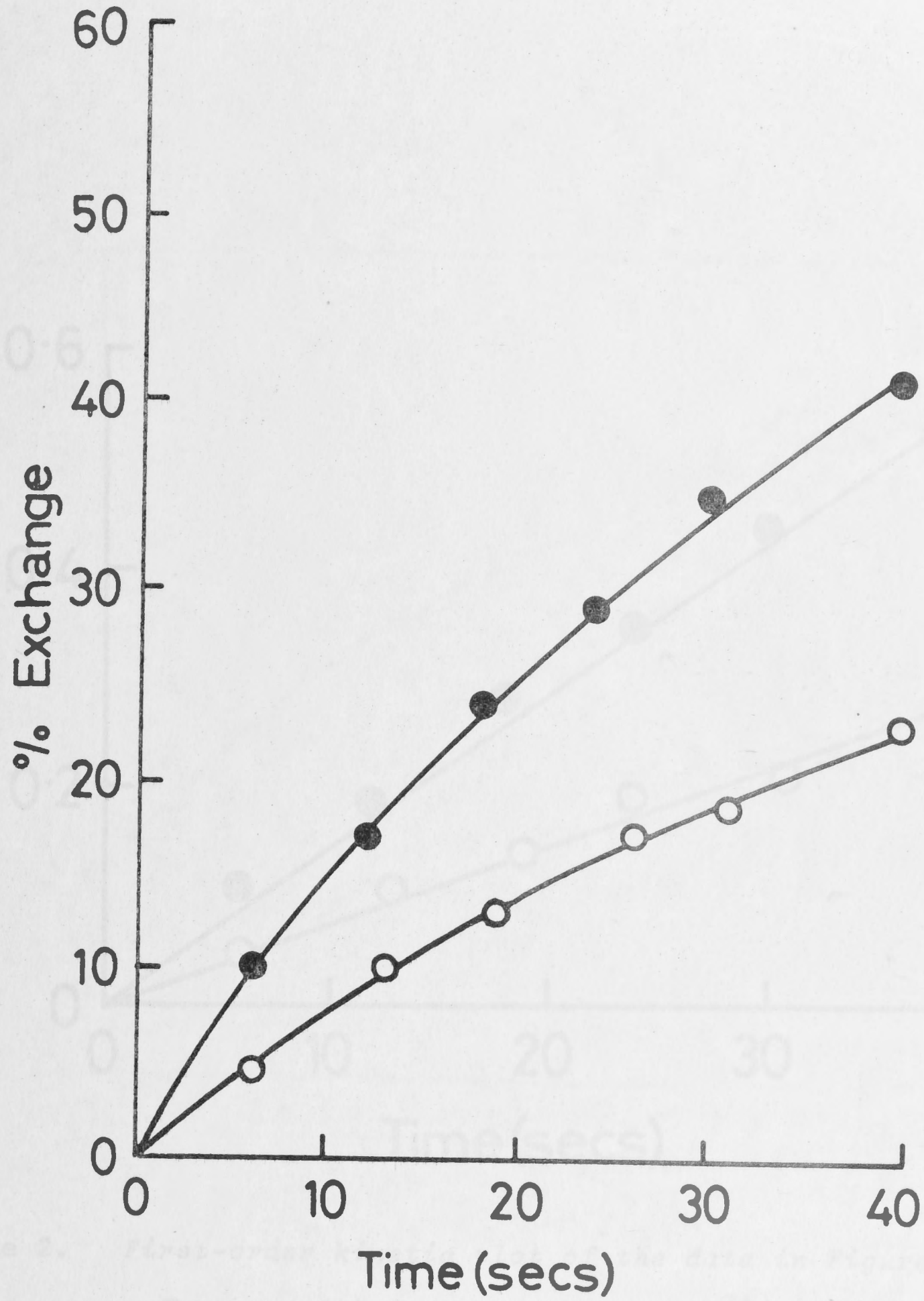


Figure 1. *Effect of Ca^{2+} on the time-course of ATP translocation.*

Mitochondria were incubated by method (a) for the times shown in the basic medium supplemented with 200μM ATP. ○ , no addition; ● , 200μM Ca^{2+}.

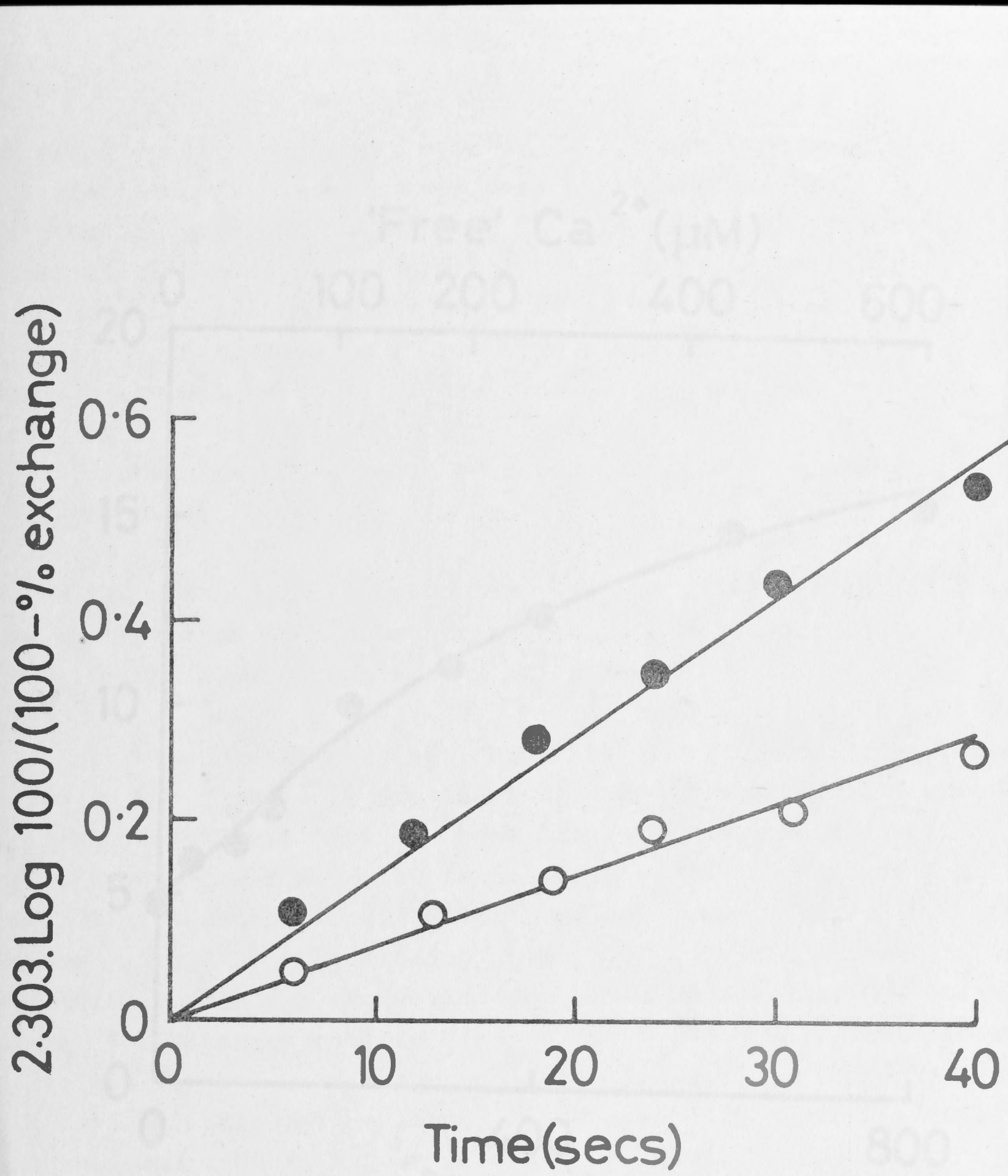


Figure 2. *First-order kinetic plot of the data in Figure 1.*

○ , no addition; ● , 200μM Ca^{2+}.

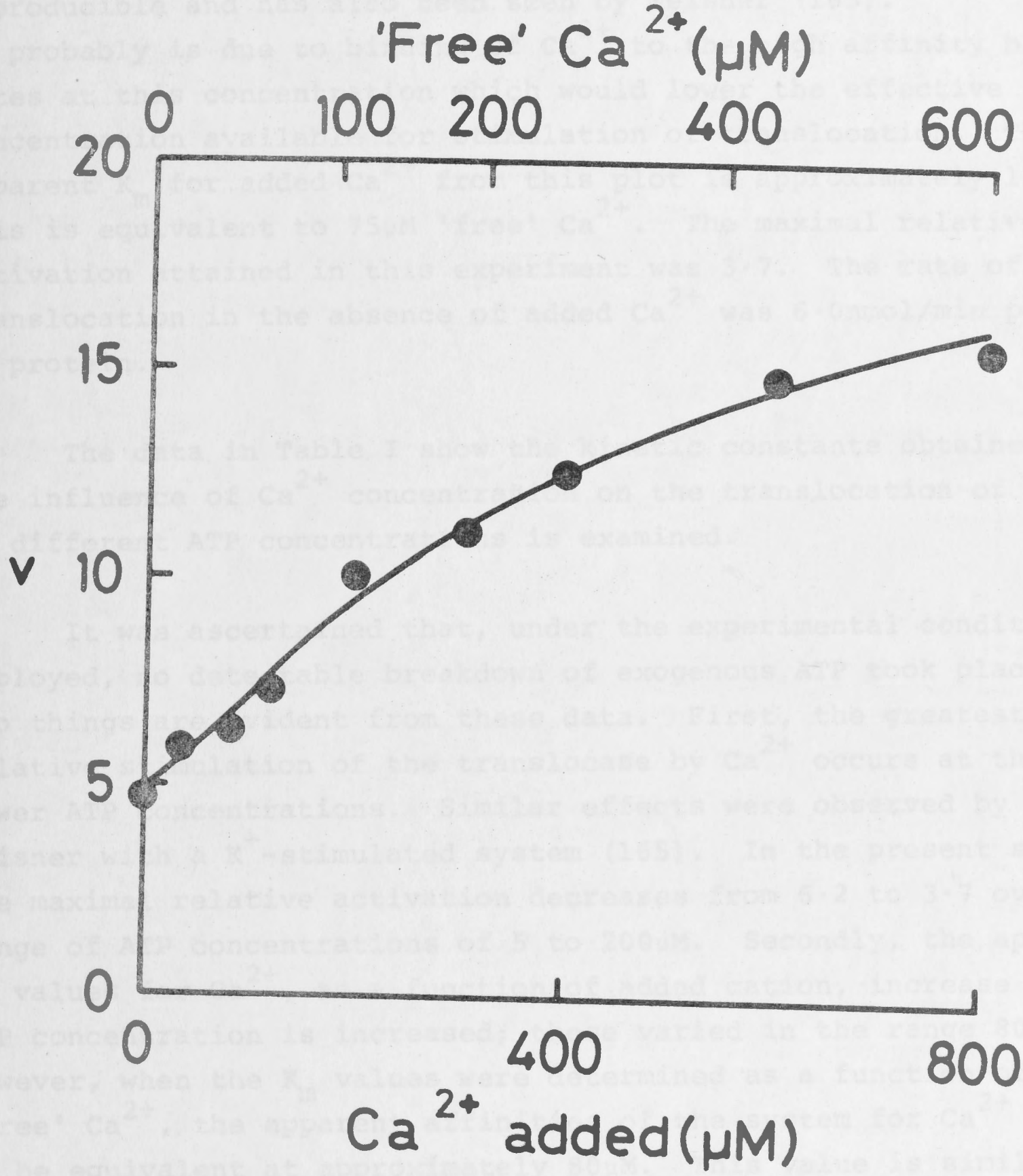


Figure 3. *Effect of Ca^{2+} concentration on the translocation of ATP.*

Mitochondria were incubated by method (b) in the basic medium supplemented with 200μM ATP and the Ca^{2+} concentrations shown. v refers to the rate of ATP translocation in nmoles/min per mg protein.

hyperbolic curve seen at the lower Ca^{2+} concentrations is highly reproducible and has also been seen by Meisner (165). It probably is due to binding of Ca^{2+} to the high affinity binding sites at this concentration which would lower the effective concentration available for stimulation of translocation. The apparent K_m for added Ca^{2+} from this plot is approximately 150μM. This is equivalent to 75μM 'free' Ca^{2+}. The maximal relative activation attained in this experiment was 3·7. The rate of ATP translocation in the absence of added Ca^{2+} was 6·0nmol/min per mg of protein.

The data in Table I show the kinetic constants obtained when the influence of Ca^{2+} concentration on the translocation of ATP at different ATP concentrations is examined.

It was ascertained that, under the experimental conditions employed, no detectable breakdown of exogenous ATP took place. Two things are evident from these data. First, the greatest relative stimulation of the translocase by Ca^{2+} occurs at the lower ATP concentrations. Similar effects were observed by Meisner with a K^+-stimulated system (165). In the present system the maximal relative activation decreases from 6·2 to 3·7 over the range of ATP concentrations of 5 to 200μM. Secondly, the apparent K_m values for Ca^{2+}, as a function of added cation, increase as the ATP concentration is increased; these varied in the range 80-160μM. However, when the K_m values were determined as a function of 'free' Ca^{2+}, the apparent affinities of the system for Ca^{2+} seem to be equivalent at approximately 80μM. This value is similar to that of the mitochondrial low affinity binding sites for Ca^{2+}. The large translocation activity at low ATP and high Ca^{2+} concentrations, when the concentration of 'free' ATP is negligible, would suggest that the $CaATP^{2-}$ complex is itself a substrate for the adenine nucleotide translocase.

Bivalent metal ion specificity for stimulation of the translocase

Table II shows the specificity of the stimulated translocation for bivalent metal ions, expressed in terms of apparent K_m values, both for added and 'free' metal ion, as well as the V_{max} Sr^{2+} appears to mimic Ca^{2+} in having both a similar K_m and V_{max}. Mg^{2+}, however, has a much lower affinity for the stimulation of

TABLE I

Kinetic constants for Ca^{2+}-stimulated translocation of ATP at several added ATP concentrations

Mitochondria were incubated by method (b) with increasing Ca^{2+} and the ATP concentrations shown. The kinetic data were determined by using double-reciprocal plots of data similar to that shown in Figure 3.

ATP concentration μM	V_{max} (maximal relative activation)	Apparent K_m for Ca^{2+} (μM)	
		'added'	'free'
5	6.2	80	78
10	5.0	90	85
50	4.1	105	84
100	3.9	130	83
200	3.7	160	75

TABLE II

Specificity of the bivalent metal ions in stimulating ATP translocation

Mitochondria were incubated by method (b) in the basic medium supplemented with 200μM ATP and various concentrations of the metal ion to be tested. The kinetic data were determined as indicated in the legend to Table I. The basal rate in the absence of added metal ions was 5·0nmol/min per mg of protein.

Metal ion tested	V_{max} (maximal relative activation)	Apparent K_m for metal ion (μM)	
		'added'	'free'
Ca^{2+}	3.8	160	75
Sr^{2+}	3.9	155	93
Mg^{2+}	2.3	690	540
Ba^{2+}	2.0	290	215
Mn^{2+}	1.2	*	*

* Not determined

ATP translocation with the K_m being about four-fold higher than that for either Ca^{2+} or Sr^{2+}. This decreased affinity is accompanied by a decreased maximal stimulation which is approximately one-half of that for Ca^{2+}. Ba^{2+} seems to be intermediate between the Ca^{2+}/Sr^{2+} pair and Mg^{2+} in enhancing the translocation of ATP, at least when expressed in terms of the K_m. However, the maximal relative activation is less than that for Ca^{2+}, Sr^{2+} or Mg^{2+}. Of the other bivalent cations tested only Mn^{2+} tends to show any stimulatory effects. In this case the maximal activation is only 1·2 at 6·4 mM added metal ion. No accurate value for the K_m can be ascribed to this cation.

Influence of Heavy metals on the translocation of adenine nucleotide

U^{2+} ions have been shown to bind to the phosphate moiety of phospholipids (227). By this action one would expect some type of effect of these ions on a membrane-localised enzyme such as the adenine nucleotide translocase. Data shown in Figure 4 indicates that U^{2+} ions inhibit the translocation of ATP and ADP in both the absence and presence of Ca^{2+}. There are several points to note concerning this inhibition. Firstly, the inhibition of ADP translocation in both cases is sigmoidal in nature whilst that of ATP is hyperbolic. Secondly, the K_i for ADP is higher than the corresponding value for ATP perhaps indicating some type of competitive inhibition in that the faster the rate of translocation the higher the concentration of a competitive-type inhibitor needed for inhibition (cf.-atractyloside). Thirdly, the K_i's in the presence of Ca^{2+} are lower than those in its absence for both ATP and ADP indicating that the Ca^{2+}-stimulated portion is being preferentially inhibited. This is borne out by reference to the insert to Figure 4 where the relative activation ratios for Ca^{2+} are plotted versus U^{2+} added. In both cases, with increasing U^{2+} concentrations, there is a decrease in this ratio such that it approaches unity. U^{2+} is known to form complexes with adenine nucleotides (69) and this might be expected to be the basis for its inhibitory properties. However, interaction of mitochondria with U^{2+} has been shown to stabilise these organelles to the extent that they become very resistant to sonication (107). This observation provides one clue to the mode of inhibition of the translocase. Under these conditions one would expect the fluidity

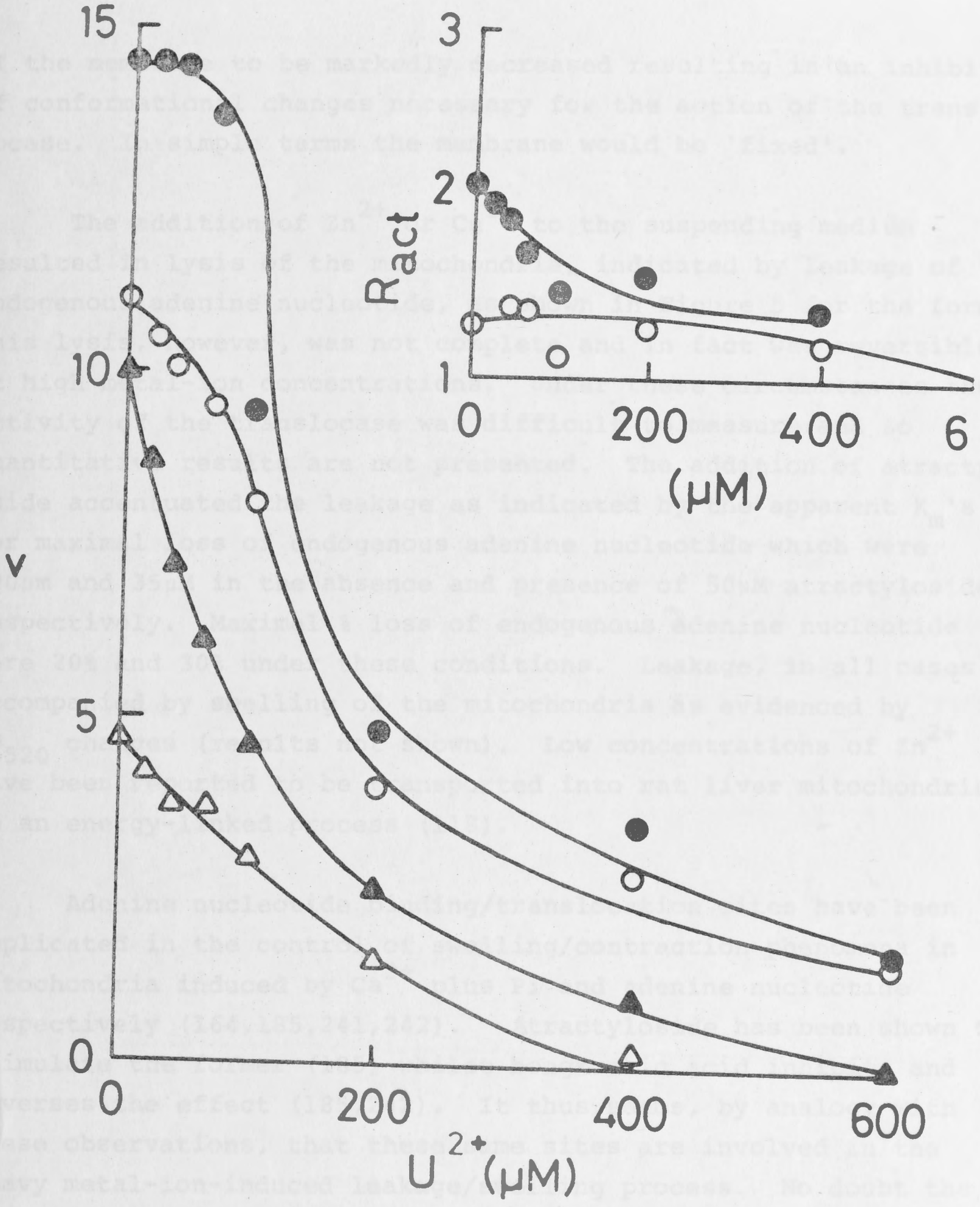


Figure 4. *Influence of U^{2+} on Ca^{2+}-stimulated adenine nucleotide translocation.*

Mitochondria were incubated by method (b) in a basic medium supplemented with 200μM ATP or ADP, various U^{2+} concentrations and 200μM Ca^{2+} as indicated. △ , ATP; ▲ , ATP plus Ca^{2+}; ○ , ADP; ● , ADP plus Ca^{2+}. v refers to the rate of adenine nucleotide translocation in nmoles/min per mg protein. Insert shows the relative activation by U^{2+} of ATP (●) and ADP (○) translocation.

of the membrane to be markedly decreased resulting in an inhibition of conformational changes necessary for the action of the translocase. In simple terms the membrane would be 'fixed'.

The addition of Zn^{2+} or Cu^{2+} to the suspending medium resulted in lysis of the mitochondria, indicated by leakage of endogenous adenine nucleotide, as shown in Figure 5 for the former. This lysis, however, was not complete and in fact was reversible at high metal-ion concentrations. Under these circumstances the activity of the translocase was difficult to measure and so quantitative results are not presented. The addition of atractyloside accentuated the leakage as indicated by the apparent K_m's for maximal loss of endogenous adenine nucleotide which were 100µm and 35µM in the absence and presence of 50µM atractyloside respectively. Maximal % loss of endogenous adenine nucleotide were 20% and 30% under these conditions. Leakage, in all cases is accompanied by swelling of the mitochondria as evidenced by OD_{520} changes (results not shown). Low concentrations of Zn^{2+} have been reported to be transported into rat liver mitochondria in an energy-linked process (118).

Adenine nucleotide binding/translocation sites have been implicated in the control of swelling/contraction phenomena in mitochondria induced by Ca^{2+} plus Pi and adenine nucleotide respectively (164,185,241,242). Atractyloside has been shown to stimulate the former (185) whilst bongkrekic acid inhibits and reverses the effect (185,242). It thus seems, by analogy with these observations, that these same sites are involved in the heavy metal-ion-induced leakage/swelling process. No doubt the leakage/swelling is brought about by some type of conformational change in the mitochondrial membrane which is inhibited when the translocase is in the 'free' state but stimulated when it is in the 'inhibited' state.

When 200µM ATP, as well as atractyloside, is added to the mitochondrial suspension there is a reversal of the potentiating effect of the atractyloside and the leakage is even lower than that in the absence of this inhibitor. Under these circumstances the toxicity of the Zn^{2+} is partially reduced by it being complexed to the ATP.

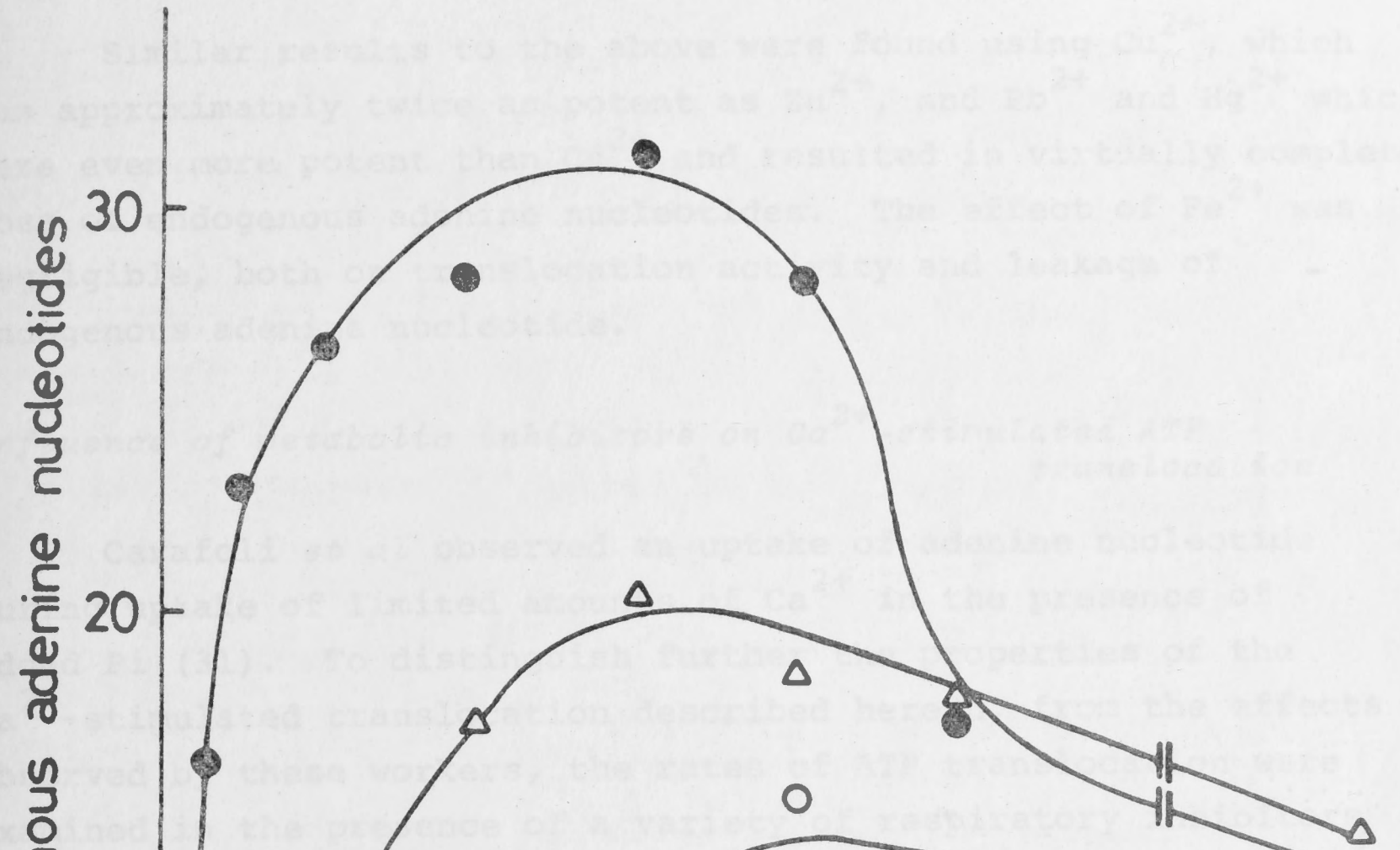


Figure 5. *Effect of Zn^{2+} concentration on loss of endogenous adenine nucleotide by mitochondria.*

Mitochondria were incubated, essentially by method (b), in a basic medium supplemented with Zn^{2+} concentrations as shown. △ , no addition; ● , 50μM atractyloside; ○ , 50μM atractyloside plus 200μM ATP.

Similar results to the above were found using Cu^{2+}, which was approximately twice as potent as Zn^{2+}, and Pb^{2+} and Hg^{2+} which were even more potent than Cu^{2+} and resulted in virtually complete loss of endogenous adenine nucleotides. The effect of Fe^{2+} was negligible, both on translocation activity and leakage of endogenous adenine nucleotide.

Influence of metabolic inhibitors on Ca^{2+}-stimulated ATP translocation

Carafoli *et al* observed an uptake of adenine nucleotide during uptake of limited amounts of Ca^{2+} in the presence of added Pi (31). To distinguish further the properties of the Ca^{2+}-stimulated translocation described herein, from the effects observed by these workers, the rates of ATP translocation were examined in the presence of a variety of respiratory inhibitors and uncouplers.

Antimycin A only slightly stimulates the translocase in either the absence or presence of Ca^{2+} (Table III). Oligomycin by itself has virtually no effect on the translocation of ATP either in the absence or presence of Ca^{2+} and of antimycin A. CCCP increases the translocation rate in both the absence and presence of Ca^{2+}. Pfaff and Klingenberg (199) observed that uncouplers of oxidative phosphorylation, such as CCCP, stimulate the translocation of ATP but not ADP. The data in Table III indicate that Ca^{2+} is not acting as an uncoupler but has a stimulatory action distinctly different from that of CCCP. Indeed, their interactions in promoting the translocation of ATP are additive. The extra addition of any or a combination of the respiratory inhibitors compensates, to a small degree, for the stimulatory effect of CCCP. The decrease in translocation activity is about 20-25% less than the maximal rate observed when only CCCP and Ca^{2+} are present together.

The influence of the polypeptide antibiotics gramicidin and valinomycin was also tested. As shown in Table IV, the addition of either antibiotic slightly increased the translocation of ATP by about 30% in the absence of Ca^{2+}, confirming previous independent studies (199). With Ca^{2+}, however, the situation is

TABLE III

Effect of metabolic inhibitors on the Ca^{2+}-stimulated ATP translocation

Mitochondria were incubated by method (a) in a basic medium supplemented with 200μM ATP. Other additions were: 200μM Ca^{2+}, 0·225μM antimycin A, 4μg of oligomycin and 5μM CCCP. The rates of translocation were calculated as shown by Pfaff *et al* (see Section B). The preincubation time was 6 minutes.

	Rate of translocation (nmol/min per mg of protein)			
	Ca^{2+} absent		Ca^{2+} present	
Inhibitor added		Relative activation		Relative activation
None	5.9	1.0	12.8	2.2
Antimycin A	6.2	1.1	14.7	2.5
Oligomycin	5.8	1.0	12.4	2.1
Antimycin A + oligomycin	6.5	1.1	15.1	2.6
CCCP	12.5	2.1	23.6	4.0
CCCP + antimycin A	12.0	2.0	22.1	3.7
CCCP + oligomycin	11.4	1.9	20.5	3.5
CCCP + antimycin A + oligomycin	10.2	1.7	18.1	3.1

TABLE IV

Effect of ion-transporting antibiotics on the Ca^{2+}-stimulated translocation of ATP

Mitochondria were incubated by method (a) in the basic medium supplemented with 200μM ATP. Other additions were: 200μM Ca^{2+}, 2μM gramicidin, 2μg of valinomycin and 5μM CCCP. The rates of translocation were calculated as indicated in the legend to Table III. The preincubation time was 6 minutes.

Inhibitor added	Rate of translocation (nmol/min per mg of protein)			
	Ca^{2+} absent		Ca^{2+} present	
		Relative activation		Relative activation
None	5.7	1.0	11.8	2.1
CCCP	10.5	1.8	20.0	3.5
Gramicidin	7.6	1.3	14.6	2.6
Gramicidin + CCCP	9.4	1.7	15.4	2.7
Valinomycin	7.3	1.3	8.7	1.5
Valinomycin + CCCP	7.7	1.4	11.3	2.0

a little more complex. The stimulation of ATP translocation produced by Ca^{2+} is negated when valinomycin, but not when gramicidin, is present. The addition of either of these antibiotics to an uncoupler-stimulated system reverses this stimulation. This is compatible with the findings of Klingenberg and Pfaff (199).

Nucelotide specificity in Ca^{2+}-stimulated translocation

Table V shows the maximal relative activation of the translocation of 200μM adenine nucleotide and the concentrations of Ca^{2+}, both 'free' and 'added', necessary to achieve half this level of stimulation. The V_{max} for ATP and dATP is approximately the same. This may be compared with that for ADP, which is about six-fold less. Since 'free' Ca^{2+} is probably the species involved in the stimulatory effect, it is not surprising that the K_m values obtained for all three nucleotides are of the order of 75μM 'free' Ca^{2+}. Other nucleotides tested included AMP,GTP, ITP,CTP,UTP and CDP. Their rates of translocation were minimal either in the presence or absence of Ca^{2+}. This specificity of the translocase for ATP and ADP is well documented (62,199,270).

As a comparison to Ca^{2+}, Mg^{2+} has differing stimulatory effects on ADP and ATP translocation. It stimulates the translocation of both adenine nucleotides to approximately the same extent whilst exhibiting a higher affinity for the ADP system.

Effect of Ca^{2+} on adenine nucleotide translocation as a function of adenine nucleotide concentration

The effect of a constant concentration of added Ca^{2+} (200μM) on the translocation of ATP, measured as a function of ATP concentration, is shown in Figure 6a. At all concentrations of ATP examined there is a significant stimulation of translocase activity. The degree of stimulation is greatest at the lowest ATP concentrations (cf. data in Table I). A similar type of experiment is shown in Figure 6b but for this the activity was examined and is expressed as a function of ADP concentration. Again the degree of stimulation is greatest at the lower nucleotide concentrations. A more direct comparison between ATP and ADP may be made when these data are replotted in a double reciprocal form to compare the K_m and V_{max} values (see Figures 7a and 7b). An

TABLE V

Nucleotide specificity of Ca^{2+} and Mg^{2+}-stimulated translocation

Mitochondria were incubated by method (b) in a basic medium supplemented with the nucleotides (present at 200μM) and various concentrations of Ca^{2+}. Values in parentheses indicate the nmol of adenine nucleotide translocated/min per mg of protein when Mg^{2+} or Ca^{2+} were not present.

	V_{max}	Apparent K_m for Ca^{2+} (μM)		V_{max}	Apparent K_a for Mg^{2+} (μM)	
Nucleotide tested	(maximal relative activation)	'added'	'free'	(maximal relative activation)	'added'	'free'
ATP	3.8.(6.0)	160	75	2.3	690	540
ADP	1.5(16.4)	90	79	2.1	250	220
dATP	4.0(1.3)	155	73	-	-	-

interesting observation was that the Lineweaver-Burk plots obtained for ATP and ADP both in the absence and presence of Ca^{2+} were biphasic. A Lineweaver-Burk plot of this general shape may represent the combined action of two saturatable transport systems, or it may alternately represent the net effect of one saturable process and one non-saturable process (163). Similar non-linearity has been observed by Pfaff *et al* (201) using an Eadie plot. Evidence to support the former possibility comes from the fact that both observed K_m values change upon the addition of Ca^{2+} ions. In the presence of 200μM Ca^{2+} the apparent K_m values for ATP decrease from 16·7μM and 67μM to 5·6μM and 32μM respectively. Similar trends were observed for ADP although the changes, especially in the case of the high affinity K_m was not nearly as marked; these fell from 9·1μM and 28μM to 6·7μM and 10·2μM respectively. There are two things to note concerning these changes. Firstly, Ca^{2+} has a greater effect on the high affinity K_m for ATP translocation compared to ADP translocation and, in actual fact, the affinity for ATP surpasses that for ADP in the presence of Ca^{2+}. Secondly, the low affinity K_m changes more markedly in the case of ADP compared to ATP and even approaches that of the high affinity K_m. Concomitantly with the increase in affinity of the systems the values for the V_{max} increase by 50% from 6·7 to 12·5 and by 15% from 11·6 to 13·4nmol/min per mg of protein for ATP and ADP respectively. Early experiments indicated that the Lineweaver-Burk plots were monophasic (235). This discrepancy was due to two factors: (a) low adenine nucleotide concentrations were not tested and (b) calculations involving incorrect values for the zero-times were performed which made the rates of translocation of intermediate concentrations of adenine nucleotide artificially high.

The data in Table VI show the effect of various added Ca^{2+} concentrations on the affinity for and maximal translocation rate of ATP. As the concentration of Ca^{2+} is increased to 0·6mM the affinity for ATP also increases. With the lower K_m this increase is of the order of 250%, from 16·1μM to 5·7μM, and for the high K_m also approximately 250%, from 77μM to 28μM. These may be compared with the K_m values for ADP, without added Ca^{2+}, of 7·3μM and 38μM. Under the same conditions the V_{max} values

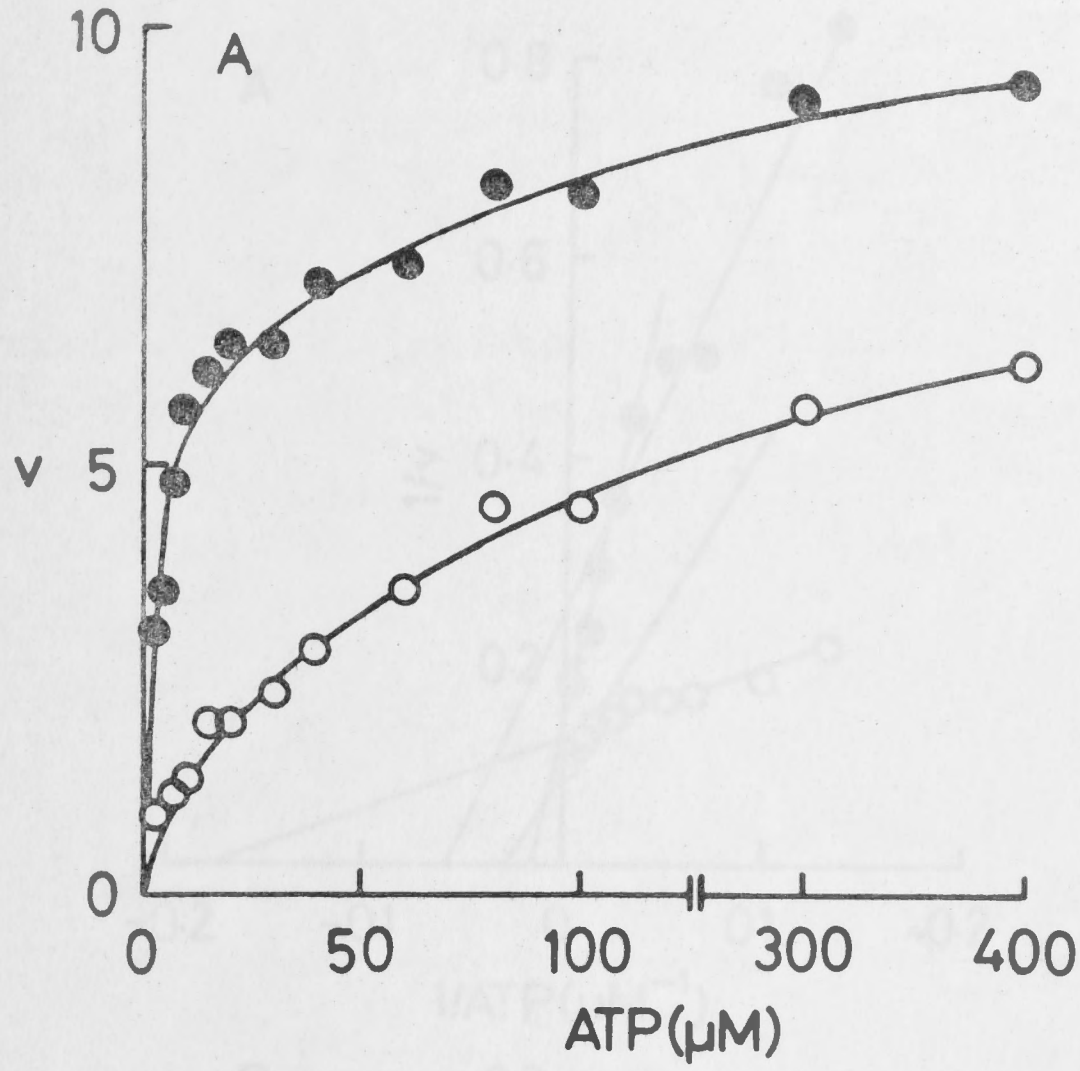
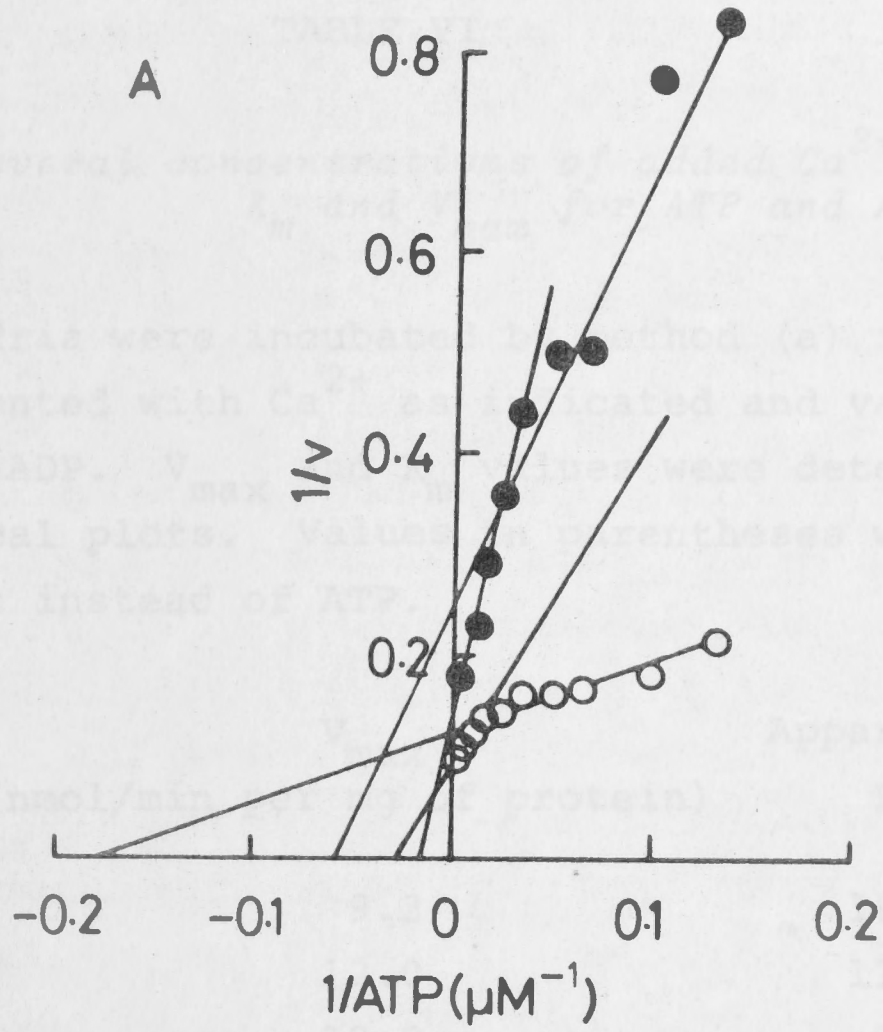


Figure 6. *Effect of Ca^{2+} on the translocation of adenine nucleotide.*

Mitochondria were incubated by method (b) in the basic medium supplemented with various concentrations of ATP and ADP and 200μM Ca^{2+} as indicated. A, ATP; B, ADP; ○ , no addition; ● , Ca^{2+}. v refers to the rate of translocation of adenine nucleotide in nmoles/min per mg protein.



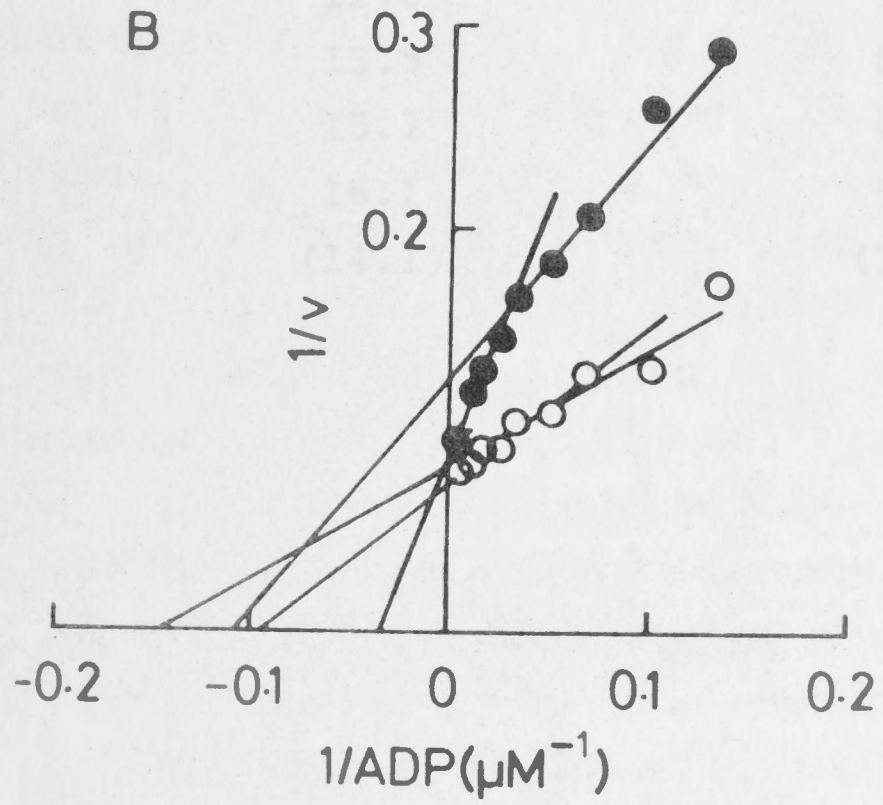


Figure 7. *Double reciprocal plots of the data in Figure 6.*

A, ATP; B, ADP; ● , no addition; ○ , 200μM Ca^{2+}.

TABLE VI

Influence of several concentrations of added Ca^{2+} on the apparent K_m and V_{max} for ATP and ADP translocation

Mitochondria were incubated by method (a) in the basic medium supplemented with Ca^{2+} as indicated and various concentrations of ATP or ADP. V_{max} and K_m values were determined by double-reciprocal plots. Values in parentheses were obtained when ADP was present instead of ATP.

Ca^{2+} added (μM)	V_{max} (nmol/min per mg of protein)	Apparent K_m for ATP (μM)	
		Low	High
0	9.3	16.1	77
50	12.0	11.0	55
100	12.8	9.4	45
200	13.5	8.3	38
400	15.1	6.7	32
600	16.1	5.7	28
(0)	(14.1)	(7.3)	(38)

increased by approximately 70% from 9·3 to 16·1nmol/min per mg of protein. This increased V_{max} value for ATP translocation is slightly higher than that observed for ADP alone.

Effect of Ca^{2+} on the competitive effects between ADP and ATP for translocation

By using a dual labelling technique it is possible to determine the simultaneous rates of translocation of ATP and ADP (see reference 199). By adopting this type of experiment and including Ca^{2+}, one is able to obtain conditions under which the addition of an equimolar concentration of ADP does not appreciably alter the ability of a particular concentration of ATP to be translocated. This effect is particularly noticeable at the lower adenine nucleotide concentrations. Thus (Table VII(i)) the addition of 50μM ADP and 80μMCa^{2+} makes little difference to the rate of translocation of 50μM ATP when compared to that rate observed in the absence of these additives; the ATP/ADP translocation ratio increased from 0·11 to 0·46. The addition of a higher concentration of Ca^{2+}, 200μM, although it further increased the translocation rate of ATP had a more marked effect on ADP translocation; in this case the translocation ratio decreased again to 0·34. Conversely the ability of Ca^{2+} to slightly stimulate the translocation of [^{14}C] ADP was reversed, when ATP was present, to a greater extent in the presence of a small concentration of Ca^{2+}(80μM) than a larger concentration (200μM). However, the addition of ADP did increase by approximately four-fold the overall translocation of adenine nucleotide as determined by the release of endogenous ^{3}H radioactivity into the incubation medium. Although the relative effect of Ca^{2+} in abolishing the ADP specificity is not as great using higher adenine nucleotide concentrations the ATP/ADP translocation ratio does still approach that observed at the lower concentrations. Thus it seems that the most favoured conditions to induce a specificity for ATP that approaches that for ADP is low adenine nucleotide and relatively low Ca^{2+} concentrations. Under normal conditions in the cell the extramitochondrial ATP/ADP ratio is high. If one compares the competitive nature of the translocase under these conditions in the absence and presence of Ca^{2+} the specificity for the entry of ADP into the mitochondrion is lost. As can be seen in Table VII(ii) the ATP/ADP translocation ratio rises from 0·72 to above unity,

TABLE VII

Effect of Ca^{2+} on the simultaneous translocation of ATP and ADP

Mitochondria were prelabelled with [^{3}H]ATP as indicated in Section B. The inward (forward exchange) and outward (back exchange) rates of adenine nucleotide translocation were determined by the method of Pfaff and Klingenberg (see Section B). Ca^{2+} and adenine nucleotide were present as shown. The different values reflect the difference in rates between the forward translocation of [^{14}C]AXP and the backward translocation of [^{3}H]-adenine nucleotide. The translocation ratio is the ratio of the rate of translocation of ATP compared to that of ADP determined in the same experiment (with the exception of B).

		Rate of translocation (nmol/min per mg of protein)			Translocation
	Reactants present	Forward (^{14}C)	Backward (^{3}H)	Difference	Ratio
A.	50µM[^{14}C]ATP	2.9	2.7	-	-
	" + 80µM Ca^{2+}	6.1	5.8	-	-
	" + 200µM Ca^{2+}	7.9	7.7	-	-
	" + 50µM ADP	0.8	8.5	7.6	0.1
	" + 50µM ADP + 80µM Ca^{2+}	2.8	8.8	6.0	0.46
	" + 50µM ADP + 200µM Ca^{2+}	3.0	11.8	8.8	0.34
	200µM[^{14}C]ATP	5.1	5.3	-	-
	" + 80µM Ca^{2+}	7.6	7.4	-	-
	" + 200µM Ca^{2+}	9.3	9.6	-	-

200μM[^{14}C]ATP	+ 200μM ADP	1.8	11.8	10.0	0.18
"	+ 200μM ADP + 80μM Ca^{2+}	3.3	10.0	6.7	0.49
"	+ 200μM ADP + 200μM Ca^{2+}	3.9	13.3	9.4	0.41
50μM[^{14}C]ADP		7.8	7.7	-	-
"	+ 80μM Ca^{2+}	8.6	8.8	-	-
"	+ 200μM Ca^{2+}	9.3	9.7	-	-
"	+ 50μM ATP	7.1	8.1	1.0	0.14
"	+ 50μM ATP + 80μM Ca^{2+}	6.2	9.0	2.8	0.45
"	+ 50μM ATP + 200μM Ca^{2+}	7.8	11.0	3.2	0.41
200μM[^{14}C]ADP		11.5	11.8	-	-
"	+ 80μM Ca^{2+}	11.9	12.2	-	-
"	+ 200μM Ca^{2+}	13.5	13.7	-	-
"	+ 200μM ATP	9.7	11.6	1.9	0.20
"	+ 200μM ATP + 80μM Ca^{2+}	6.6	9.7	3.1	0.47
"	+ 200μM ATP + 200μM Ca^{2+}	8.6	12.6	4.0	0.46
B. 100μM[^{14}C]ATP		3.7	-	-	-
"	+ 200μM Ca^{2+}	8.2	-	-	-
"	+ 20μM ADP	2.6	-	-	-
"	20μM ADP + 200μM Ca^{2+}	4.9	-	-	-

20μM[^{14}C]ADP	4.7	-	-	-
" + 200μM Ca^{2+}	7.0	-	-	-
" + 100μM ATP	3.6	-	-	-
" + 100μM ATP + 200μM Ca^{2+}	3.2	-	-	-

Translocation ratios for 100μM ATP plus 20μM ADP in the absence and presence of 200μM Ca^{2+} are 0·72 ($\frac{2.6}{3.6}$) and 1·69 ($\frac{4.9}{3.2}$), respectively.

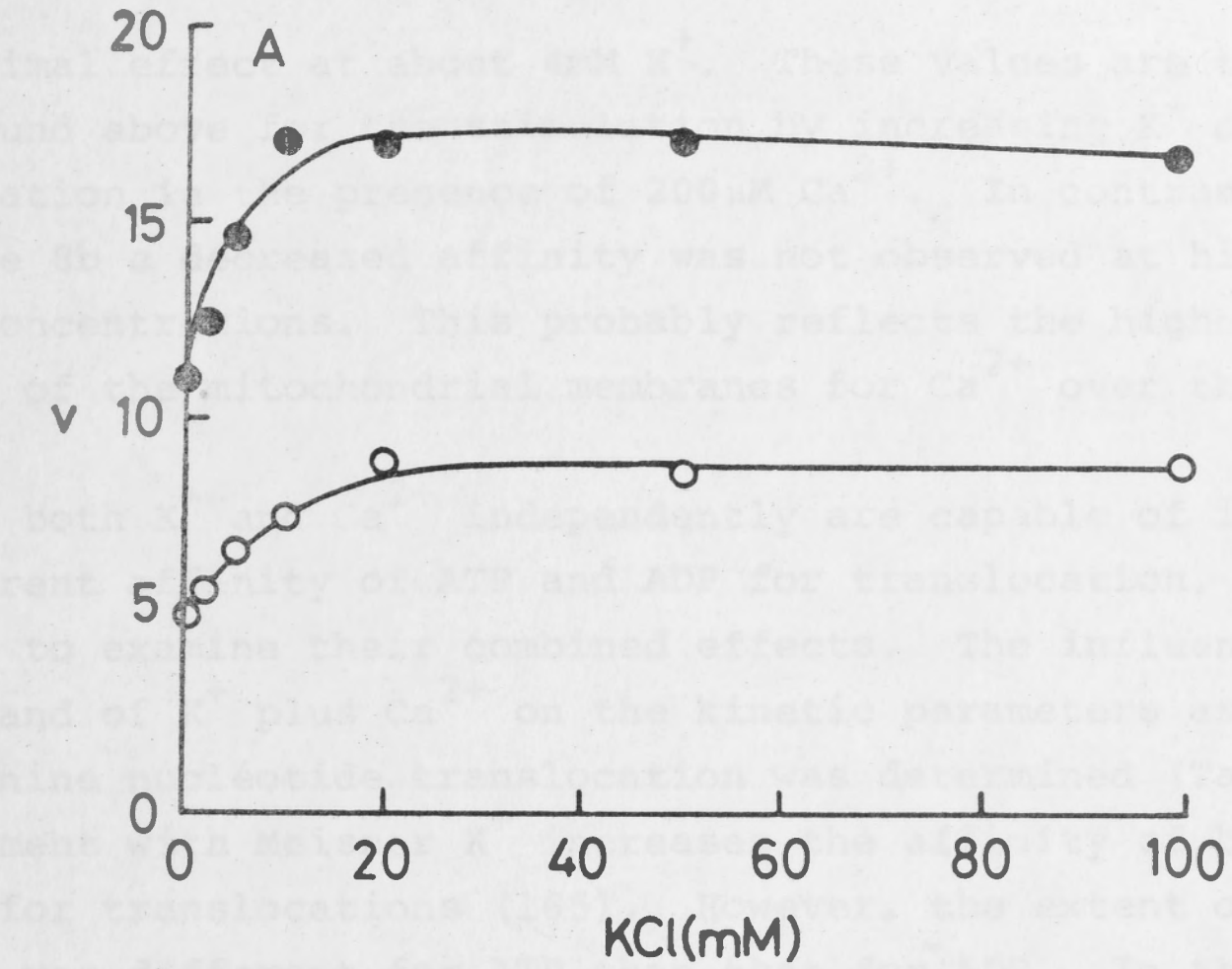
1·69, when 200μM Ca^{2+} is added. These latter experiments were performed using the forward exchange only and testing the effect of the complementary nucleotide on the rate of translocation.

Influence of Ca^{2+} on K^+-stimulated translocation of ATP

Meisner has shown that added K^+ enhances the translocation of both ATP and ADP by isolated heart and liver mitochondria (165). The data of Figure 8a confirm this observation for ATP translocation. They also show that the addition of 200μM Ca^{2+} both further stimulates the translocation of ATP and appears to increase the affinity of the system for K^+. The respective interactions of K^+ and Ca^{2+} with the translocase appear to be additive. At higher K^+ concentrations a slight decrease in the stimulation is observable. Data in Figure 8b summarises the apparent affinities of the system for K^+ as a function of the Ca^{2+} ion added. Up to approximately 200μM Ca^{2+} there is an increased affinity for K^+; the K_m drops by 33% from 6mM to 4mM. However, the addition of Ca^{2+} concentrations above this value of 200μM resulted in a marked diminuition in the affinity with the K_m rising to 9mM at 0·8mM Ca^{2+}. It appears that under these conditions the additive effects of K^+ and Ca^{2+} are decreased probably due to an interaction between K^+ and Ca^{2+} effector sites. This is corroborated by results not shown which indicate that the maximal relative activation decreases rapidly as the Ca^{2+} concentration is increased. K^+ also stimulates the translocation of ADP. In a typical experiment (results not shown) the maximal relative activation was approximately 1·6 with half-maximal effect at 2·6mM.

Influence of K^+ on Ca^{2+}-stimulated translocation of ATP

Addition of 20mM K^+ enhances the Ca^{2+}-stimulated ATP translocation over the entire Ca^{2+} concentration range examined (Figure 9a). The added K^+ also appears to increase the apparent affinity of ATP translocation for Ca^{2+}. This was confirmed in an experiment (Figure 9b) in which a number of Ca^{2+} concentration curves were determined in the presence of different amounts of K^+. As the K^+ concentration was increased from 0 to 100mM, the apparent K_m for added Ca^{2+} decreased from 160 to 50μM. This is equivalent to 'free' Ca^{2+} concentrations of 75μM and 23μM respectively. The maximal effect was achieved by 20mM K^+ with a



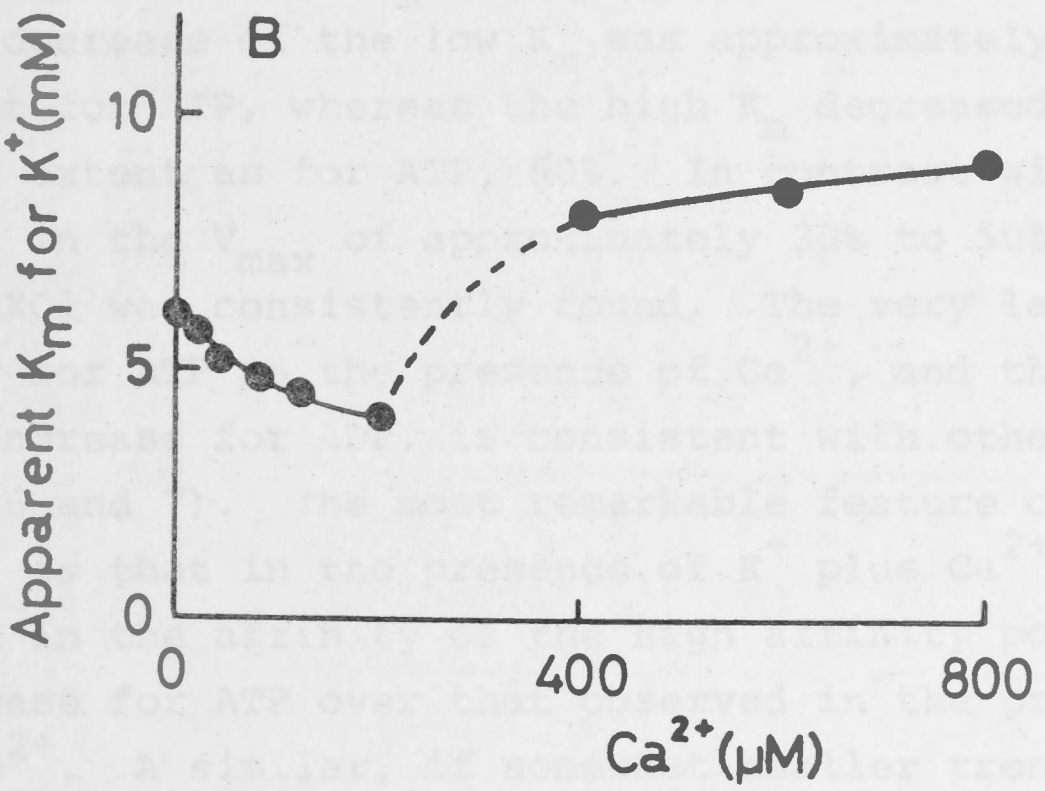


Figure 8. *Influence of Ca^{2+} on K^+-stimulated translocation of ATP.*

Mitochondria were incubated by method (b) in a basic medium supplemented with 200μM ATP, various concentrations of K^+ and 200μM Ca^{2+} as indicated. The solute concentration was kept constant at 200 milliosmolar by altering the amount of sucrose added. A, ○ , no addition; ● ,Ca^{2+}; B, the apparent K_m values for K^+ were calculated from double-reciprocal plots redrawn from a series of K^+ concentration curves as in A. v refers to the rate of ATP translocation in nmoles/min per mg protein.

half-maximal effect at about 4mM K^+. These values are the same as those found above for the stimulation by increasing K^+ of ATP translocation in the presence of 200μM Ca^{2+}. In contrast to data in Figure 8b a decreased affinity was not observed at higher cation concentrations. This probably reflects the higher overall affinity of the mitochondrial membranes for Ca^{2+} over that for K^+.

As both K^+ and Ca^{2+} independently are capable of increasing the apparent affinity of ATP and ADP for translocation, it was of interest to examine their combined effects. The influence of K^+, of Ca^{2+} and of K^+ plus Ca^{2+} on the kinetic parameters associated with adenine nucleotide translocation was determined (Table VIII). In agreement with Meisner K^+ increases the affinity of both ATP and ADP for translocations (165). However, the extent of the increase was different for ATP than that for ADP. In the case of the former the low K_m decreased approximately 75% from 15·6 to 8·9μM whilst the high K_m decreased by 55% from 100 to 45μM. With ADP the decrease of the low K_m was approximately 20%, much lower than that for ATP, whereas the high K_m decreased by approximately the same extent as for ATP, 60%. In contrast with Meisner an increase in the V_{max} of approximately 30% to 50% in the presence of 20mM KCl was consistently found. The very large increase in affinity for ATP in the presence of Ca^{2+}, and the correspondingly slight increase for ADP, is consistent with other data (see Figures 6 and 7). The most remarkable feature of the results, however, is that in the presence of K^+ plus Ca^{2+} there was a decrease in the affinity of the high affinity portion of the translocase for ATP over that observed in the presence of either K^+ or Ca^{2+}. A similar, if somewhat smaller trend, is also obersved for ADP. The low affinity portion of the translocation process did, however, increase in affinity for both ATP and ADP under these conditions. The V_{max} for ATP increases by 150% over the control to 18·1nmol/min per mg of protein whereas that for ADP increases by only 60% to 19·9nmol/min per mg of protein.

In other control experiments employing a combination of ^3H_2O, [^{14}C] sucrose and the light-scattering technique, no swelling of the mitochondria occurred under the conditions of the translocase assay in the absence or presence of KCl.

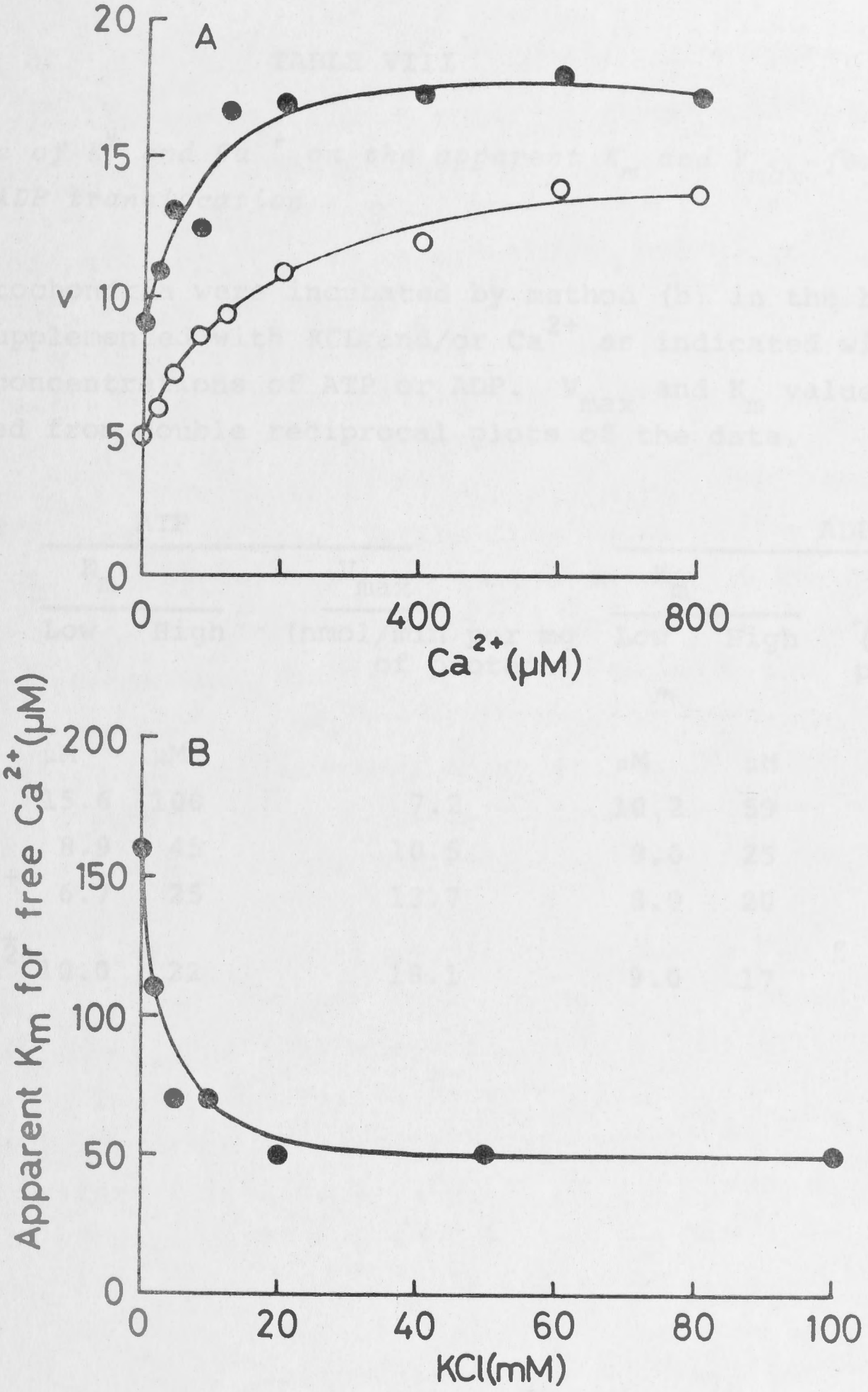


Figure 9. *Influence of K^+ on Ca^{2+}-stimulated translocation of ATP.*

Mitochondria were incubated by method (b) in a basic medium supplemented with 200μM ATP, various concentrations of Ca^{2+} and 20mM K^+ as indicated. The solute concentration was kept constant at 200 milliosmolar by altering the amount of sucrose added. A, ○ , no addition; ● , 20mM K^+; B, the apparent K_m values for Ca^{2+} were calculated from double-reciprocal plots redrawn from a series of Ca^{2+} concentration curves as in A. v refers to the rate of ATP translocation in nmoles/min per mg protein.

TABLE VIII

Influence of K^+ and Ca^{2+} on the apparent K_m and V_{max} for ATP and ADP translocation

Mitochondria were incubated by method (b) in the basic medium supplemented with KCL and/or Ca^{2+} as indicated with various concentrations of ATP or ADP. V_{max} and K_m values were determined from double reciprocal plots of the data.

	ATP			ADP		
	K_m		V_{max}	K_m		V_{max}
	Low	High	(nmol/min per mg of protein)	Low	High	(nmol/min per mg of protein)
Addition	µM	µM		µM	µM	
None	15.6	100	7.2	10.2	59	12.5
20mM KCl	8.9	45	10.5	9.0	25	16.0
200µM Ca^{2+}	6.7	25	13.7	8.0	20	14.3
20mM Kcl + 200µM Ca^{2+}	10.0	22	18.1	9.0	17	19.9

Influence of K^+ on Mg^{2+}-stimulated translocation of ATP

In view of the difference in affinity for and extent of Ca^{2+}- and Mg^{2+}-stimulated translocation of ATP it was considered of interest to test the effect of the addition of K^+ ions on the latter. The results of such an experiment are shown in Figure 10a,b. Contrary to the effect of K^+ on the Ca^{+2}-stimulated translocation of ATP (see Figure 9) the addition of 20mM K^+ to the Mg^{2+}-stimulated system resulted in an inhibition of the stimulation. In actual fact the stimulatory effect was reversed at higher Mg^{2+} concentrations by approximately 25%. When 200μm Ca^{2+} was also present in the incubation medium two things were evident. Firstly, the stimulatory action of the Mg^{2+} was decreased in the presence of the Ca^{2+}; maximal relative stimulation was 2·3 in the absence and 1·6 in the presence of Ca^{2+}. Secondly, at low Mg^{2+} concentrations, up to 0·3mM, the presence of 20mM K^+ did potentiate the slight stimulatory action of the Mg^{2+} ions. Figure 10c,d shows similar results to those described above but in this case the adenine nucleotide being translocated was ADP.

Decreases in the rates of translocation in the presence of K^+ ions were not as apparent in the case of Mg^{2+}-stimulated ADP translocation (Figure 10b) when compared to that of ATP; at 3mM Mg^{2+} the inhibition varied between 10% and 15%. As in the case of ATP the maximum relative activation by Mg^{2+} (K^+-free system) fell from 2·0 to 1·5 in the presence of Ca^{2+} ions. Unlike ATP no maxima was apparent in the translocation rate in the presence of both K^+ and Ca^{2+}, only inhibition.

Effect of La^{3+} on Ca^{2+}-stimulated adenine nucleotide translocation

In efforts to further define the nature of the stimulation by Ca^{2+}, studies were carried out with the rare earth cation La^{3+}. This ion has proved to be a useful probe for studying interactions of Ca^{2+} with proteins, lipids and membranes in general (16,150,252) as well as with Ca^{2+}-induced energy-dependent processes in mitochondria (149,167,169).

The influence of La^{3+} on the translocation of ATP and ADP with and without added Ca^{2+} is shown in Figure 11. The following effects can be seen: (a) only that portion of ATP translocation

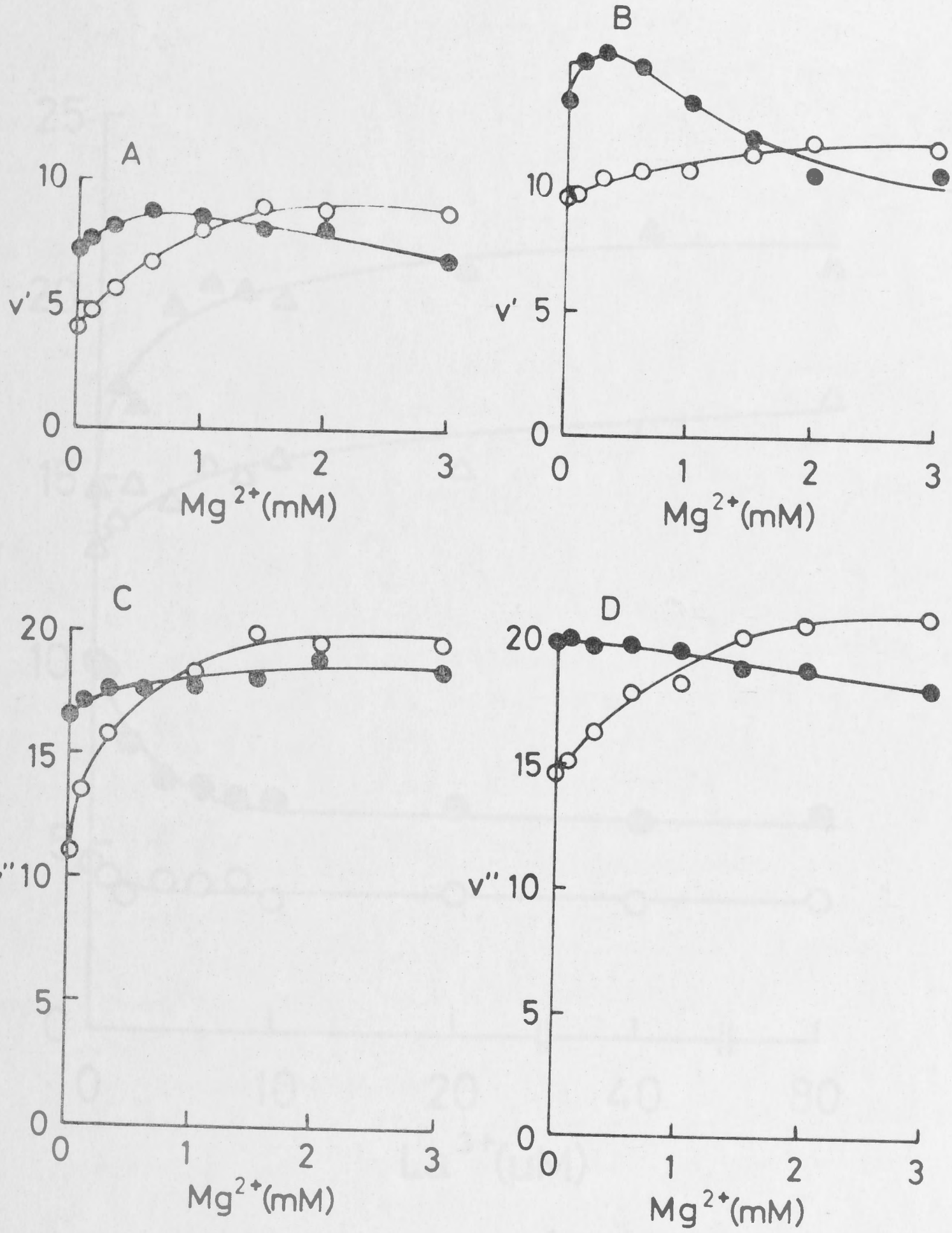


Figure 10. *Influence of Ca^{2+} and K^{+} on Mg^{2+}-stimulated adenine nucleotide translocation.*

Mitochondria were incubated by method (b) in a basic medium supplemented with 200μM adenine nucleotide and various concentrations of Mg^{2+}. Ca^{2+} and K^{+} when present were at concentrations of 200μM and 20mM, respectively. The solute concentration was kept constant at 200 milliosmolar by altering the amount of sucrose added. A, ATP; B, ATP plus Ca^{2+}; C, ADP; D, ADP plus Ca^{2+}. Open symbols, minus K^{+}; closed symbols, plus 20mM K^{+}. v' and v" refer to the rate of translocation of ATP and ADP, respectively, in nmoles/min per mg protein.

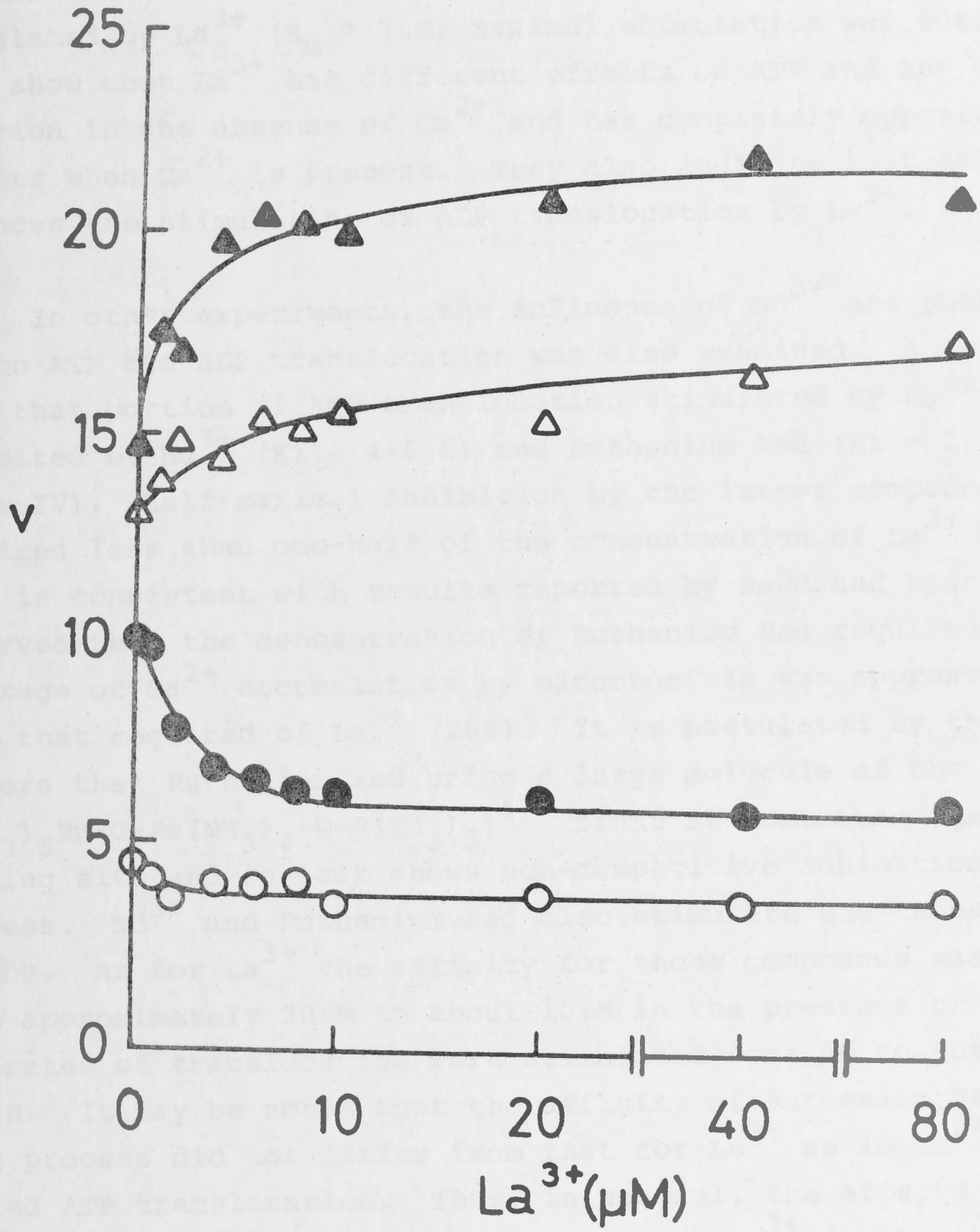


Figure 11. *Influence of La^{3+} on Ca^{2+}-stimulated adenine nucleotide translocation.*

Mitochondria were incubated by method (b) in a basic medium supplemented with 200μM ATP or ADP, various La^{3+} concentrations and 200μM Ca^{2+} as indicated. ○ , ATP; ● , ATP plus Ca^{2+}; △ , ADP; ▲ , ADP plus Ca^{2+}. v refers to the rate of translocation of adenine nucleotide in nmoles/min per mg protein.

which is stimulated by Ca^{2+} is inhibited by La^{3+} (Ki = 3·5μM); (b) ADP translocation in the absence of added Ca^{2+} is slightly stimulated by La^{3+} (K_m = 7μM; maximal stimulation was 60%). These data show that La^{3+} has different effects on ATP and ADP translocation in the absence of Ca^{2+} and has completely opposite effects when Ca^{2+} is present. They also indicate that added Ca^{2+} enhances the stimulation of ADP translocation by La^{3+}.

In other experiments, the influence of Nd^{3+} and Ruthenium Red on ATP and ADP translocation was also examined. As with La^{3+}, only that portion of ATP translocation stimulated by Ca^{2+} was inhibited by Nd^{3+} (Ki = 4·5μM) and Ruthenium Red (Ki = 1·5μM) Table IV). Half-maximal inhibition by the latter compound required less than one-half of the concentration of La^{3+} and Nd^{3+}. This is consistent with results reported by Reed and Bygrave who observed that the concentration of Ruthenium Red required for blockage of Ca^{2+} accumulation by mitochondria was approximately half that required of La^{3+} (209). It is postulated by these workers that Ruthenium Red being a large molecule of the structure $[(NH_3)_5Ru\text{-}O\text{-}Ru(NH_3)_4\text{-}O\text{-}R(NH_3)_5]^{6+}$ binds at more than one Ca^{2+} binding site and in fact shows non-competitive inhibition of this process. Nd^{3+} and Ruthenium Red also stimulate the translocation of ADP. As for La^{3+} the affinity for these compounds was increased from approximately 30μM to about 10μM in the presence of Ca^{2+} ions. The rates of translocation were stimulated some 50 to 60% in all cases. It may be noted that the affinity of Ruthenium Red for this process did not differ from that for La^{3+} as in Ca^{2+}-stimulated ATP translocation. Thus, in general, the effects on adenine nucleotide translocation seen with La^{3+} in the absence and presence of Ca^{2+} are seen also with other rare earth cations and Ca^{2+} antagonists.

Data in Table X show that the K_i for La^{3+} inhibition of Ca^{2+}-stimulated ATP translocation increases as the Ca^{2+} concentration increases. These findings further suggest that Ca^{2+} and La^{3+} compete for a common (binding) site in the vicinity of the translocase. At the higher Ca^{2+} concentrations the curves relating % inhibition and added La^{3+} concentration were slightly sigmoidal.

TABLE IX

Comparison of La^{3+}, Nd^{3+} and Ruthenium Red as effectors of Ca^{2+}-stimulated adenine nucleotide translocation

Mitochondria were incubated and the data analysed essentially as indicated in the legend to Figure 11.

	ATP	ADP			
	K_i	minus Ca^{2+}		plus Ca^{2+}	
		K_a	maximal stim.	K_a	maximal stim.
Compound tested	µM	µM	%	µM	%
La^{3+}	5.5	25	55	10	60
Nd^{3+}	4.5	28	60	10	50
Ruthenium Red	1.5	30	55	8	58

TABLE X

Effect of Ca^{2+} concentration on the inhibitor constant for La^{3+} of Ca^{2+}-stimulated translocation of ATP

Mitochondria were incubated and the data analysed essentially as indicated in the legend to Figure 11 except that only ATP translocation was tested.

Ca^{2+} concentration	K_i for La^{3+}
µM	µM
100	2.4
200	4.0
400	12.5
1000	18.0

Previous results (see Tables III and IV) indicate that the mechanism whereby Ca^{2+} ions stimulate ATP translocation is different from that involving uncouplers of oxidative phosphorylation such as CCCP. Data shown in Figure 12 strongly supports this contention. Here it is seen that, in contrast to Ca^{2+}-stimulated ATP translocation (see Figure 11) uncoupler-stimulated ATP translocation is virtually unaffected by La^{3+} concentrations up to 80μM.

K^+ ions stimulate ATP translocation in rat liver mitochondria in a manner which is different from that whereby Ca^{2+} stimulates ATP translocation as shown by the additivity of the K^+ and Ca^{2+} effects (see Figures 8 and 9). Figure 13 shows results from an experiment in which the effect of La^{3+} on ATP translocation was studied with and without added K^+ and Ca^{2+}. La^{3+} clearly has no effect on the K^+-stimulated portion of ATP translocation but at the same time inhibits the Ca^{2+}-stimulated portion. Moreover the presence of K^+ does not alter the affinity of the inhibition for La^{3+} (Ki = 5μM in the absence and presence of K^+).

One feature of the La^{3+} inhibition plots was that under all conditions 100% inhibition of the Ca^{2+}-stimulated portion of ATP translocation was never attained. Several reasons are forthcoming to explain this phenomenon. The most obvious one is that the inhibition, being competitive in nature, was not complete due to use of too low La^{3+} concentrations. Secondly, a portion of the Ca^{2+}-stimulated ATP translocation may be due to the formation of the CaATP complex. If higher concentrations of La^{3+}, over 200μM, were employed in the incubation medium the interpretation of the results was difficult due to an observed inhibition of the translocation even in the absence of Ca^{2+}. The inhibition is probably tied up with observed 'clumping' of mitochondria under the effect of these high La^{3+} concentrations. This 'clumping' is probably a consequence of 'cross-linking' of the mitochondrial membranes which decreases the surface area and thus limits the accessibility of the adenine nucleotide to the membrane and the translocase. Another explanation for this inhibition is that the LaATP complex is not a substrate for the translocase but this is difficult to reconcile with the observed non-inhibition at lower La^{3+} concentrations (see Figure 11).

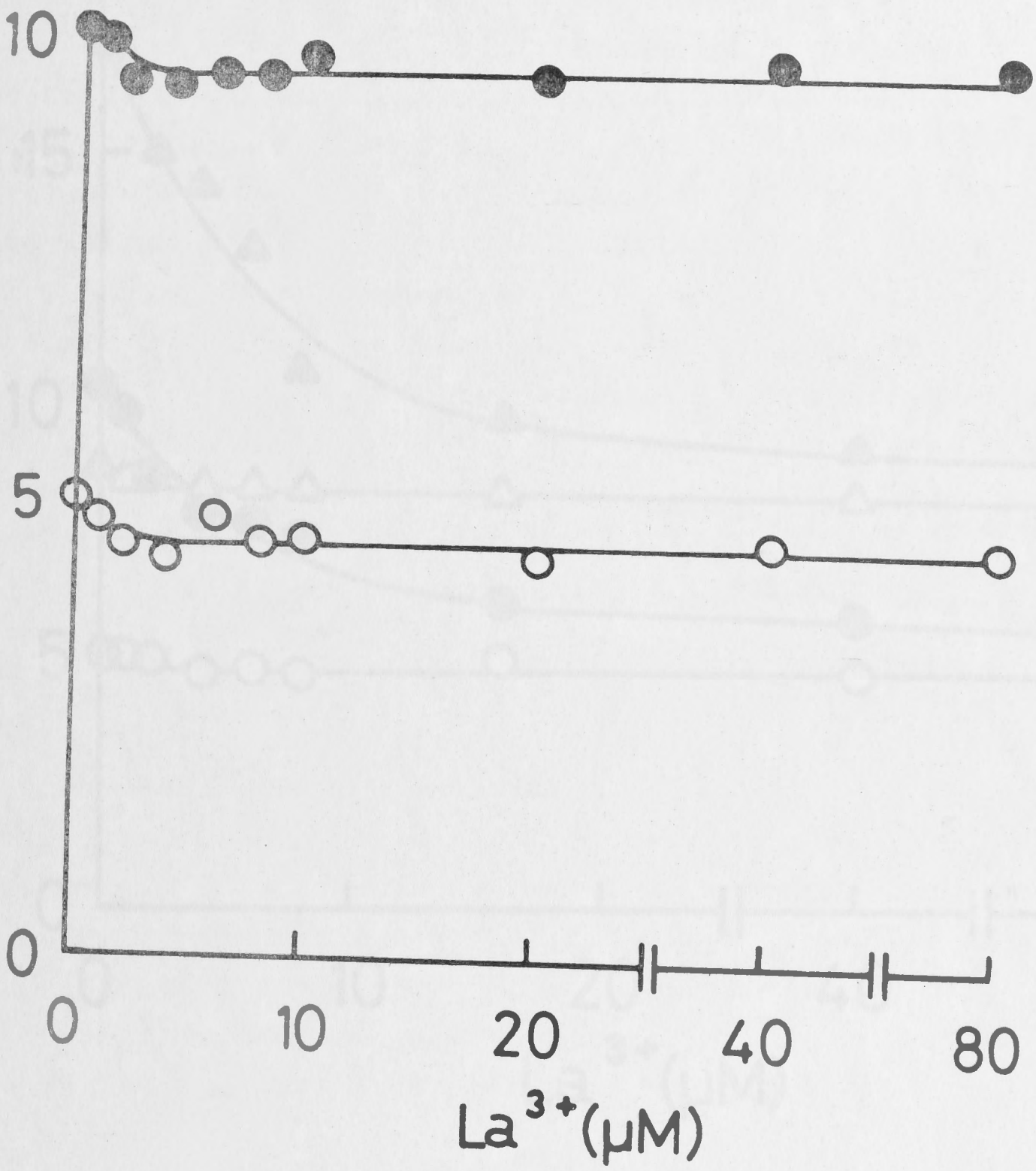


Figure 12. *Influence of La^{3+} on CCCP-stimulated ATP translocation.*

Mitochondria were incubated by method (b) in a basic medium supplemented with 200μM ATP, various concentrations of La^{3+} and 2μM CCCP as indicated. ○ , no addition ; ● , CCCP. v refers to the rate of translocation of ATP in nmoles/min per mg protein.

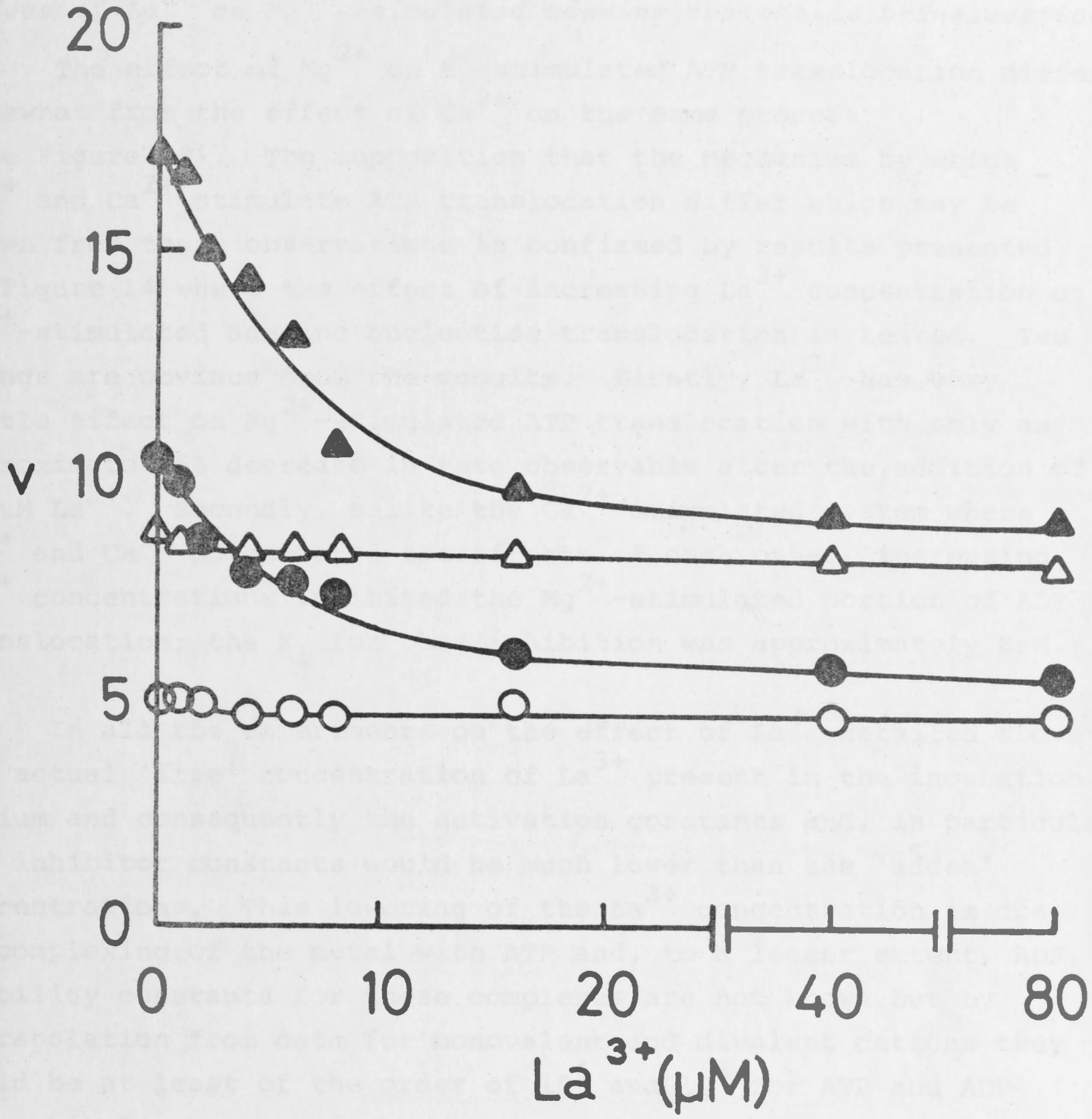


Figure 13. *Comparison of the influence of La^{3+} on Ca^{2+}-stimulated and K^+-stimulated ATP translocation.*

Mitochondria were incubated by method (b) in a basic medium supplemented with 200μM ATP various La^{3+} concentrations, 200μM Ca^{2+} and 20mM K^+ as indicated. ○ , no additions; ● , Ca^{2+}; △ , K^+; ▲ , Ca^{2+} plus K^+. v refers to the rate of translocation of ATP in nmoles/min per mg protein.

Effect of La^{3+} on Mg^{2+}-stimulated adenine nucleotide translocation

The effect of Mg^{2+} on K^{+}-stimulated ATP translocation differs somewhat from the effect of Ca^{2+} on the same process (see Figure 10). The supposition that the mechanism by which Mg^{2+} and Ca^{2+} stimulate ATP translocation differ which may be drawn from these observations is confirmed by results presented in Figure 14 where the effect of increasing La^{3+} concentration on Mg^{2+}-stimulated adenine nucleotide translocation is tested. Two things are obvious from the results. Firstly, La^{3+} has very little effect on Mg^{2+}-stimulated ATP translocation with only an approximate 25% decrease in rate observable after the addition of 40μM La^{3+}. Secondly, unlike the Ca^{2+}-stimulated system where La^{3+} and Ca^{2+} potentiated the effects of each other, increasing La^{3+} concentrations inhibited the Mg^{2+}-stimulated portion of ADP translocation; the K_i for this inhibition was approximately 8μM.

In all the experiments on the effect of La^{3+} detailed above the actual 'free' concentration of La^{3+} present in the incubation medium and consequently the activation constants and, in particular the inhibitor constants would be much lower than the 'added' concentrations. This lowering of the La^{3+} concentration is due to complexing of the metal with ATP and, to a lesser extent, ADP. Stability constants for these complexes are not known but by extrapolation from data for monovalent and divalent cations they would be at least of the order of 10^6 and 10^5 for ATP and ADP respectively.

Influence of preincubation time with Ca^{2+} on Ca^{2+}-stimulated adenine nucleotide translocation

Data in Figure 15 show the effect on adenine nucleotide translocation of preincubating mitochondria in the presence of 200μM Ca^{2+} and, as a control, in the absence of added Ca^{2+}. As can be seen from Figure 15a in the control case there is little change in the rate of ATP translocation up to 20 min. When Ca^{2+} is present increasing time of preincubation results in a decrease in the Ca^{2+}-stimulated portion of the ATP translocation of up to 75% after 20 min; the half-time is about 5 min. Levels of Ca^{2+} accumulated in the mitochondria during this time course are shown in Figure 15b. If 0·2mM succinate is also present in the incubation medium the responses to increasing preincubation time

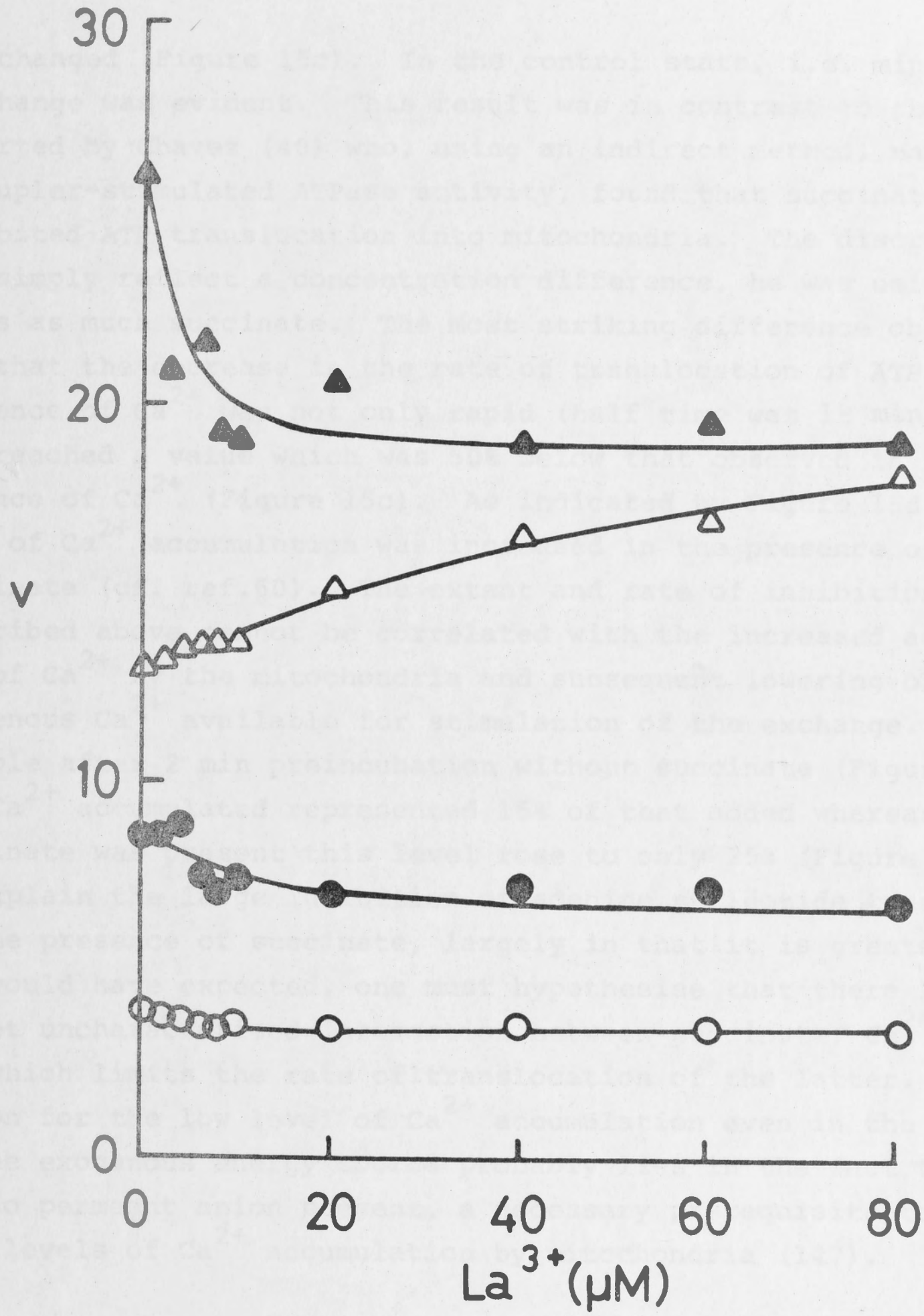


Figure 14. *Influence of La^{3+} on Mg^{2+}-stimulated adenine nucleotide translocation.*

Mitochondria were incubated by method (b) in a basic medium supplemented with 200μM ATP, or ADP, various La^{3+} concentrations and 4mM Mg^{2+} as indicated. ○ , ATP; ● , ATP plus Mg^{2+}; △ , ADP; ▲ , ADP plus Mg^{2+}. v refers to the rate of translocation of adenine nucleotide in nmoles/min per mg protein.

are changed (Figure 15c). In the control state, i.e. minus Ca^{2+}, no change was evident. This result was in contrast to those reported by Chavez (40) who, using an indirect method, namely uncoupler-stimulated ATPase activity, found that succinate inhibited ATP translocation into mitochondria. The discrepancy may simply reflect a concentration difference, he was using 20 times as much succinate. The most striking difference observed was that the decrease in the rate of translocation of ATP in the presence of Ca^{2+} was not only rapid (half time was 1½ minutes) but reached a value which was 50% below that observed in the absence of Ca^{2+} (Figure 15c). As indicated by Figure 15d the rate of Ca^{2+} accumulation was increased in the presence of succinate (cf. ref.60). The extent and rate of inhibition described above cannot be correlated with the increased accumulation of Ca^{2+} by the mitochondria and subsequent lowering of the exogenous Ca^{2+} available for stimulation of the exchange. For example after 2 min preincubation without succinate (Figure 15b) the Ca^{2+} accumulated represented 15% of that added whereas when succinate was present this level rose to only 25% (Figure 15d). To explain the large inhibition of adenine nucleotide translocation in the presence of succinate, largely in that it is greater than one would have expected, one must hypothesise that there is some as yet uncharacterised interaction between succinate, Ca^{2+} and ATP which limits the rate of translocation of the latter. The reason for the low level of Ca^{2+} accumulation even in the presence of the exogenous energy source probably lies in the fact that there was no permeant anion present, a necessary prerequisite to support high levels of Ca^{2+} accumulation by mitochondria (147).

Inhibition of the availability of energy by the addition of the uncoupler CCCP results in virtually no change in the rate of translocation of ATP without Ca^{2+} in the preincubation medium (Figure 15e). This indicates that, at least up to 20 min, there is no breakdown of the mitochondrial membrane and no significant loss in exchangeable adenine nucleotide. This latter point was verified by the observation that no appreciable increase in the zero-time values occured. When 200μM Ca^{2+} is present in the medium there is an approximate 10% decrease in the rate of translocation of ATP at higher times of preincubation. This slight

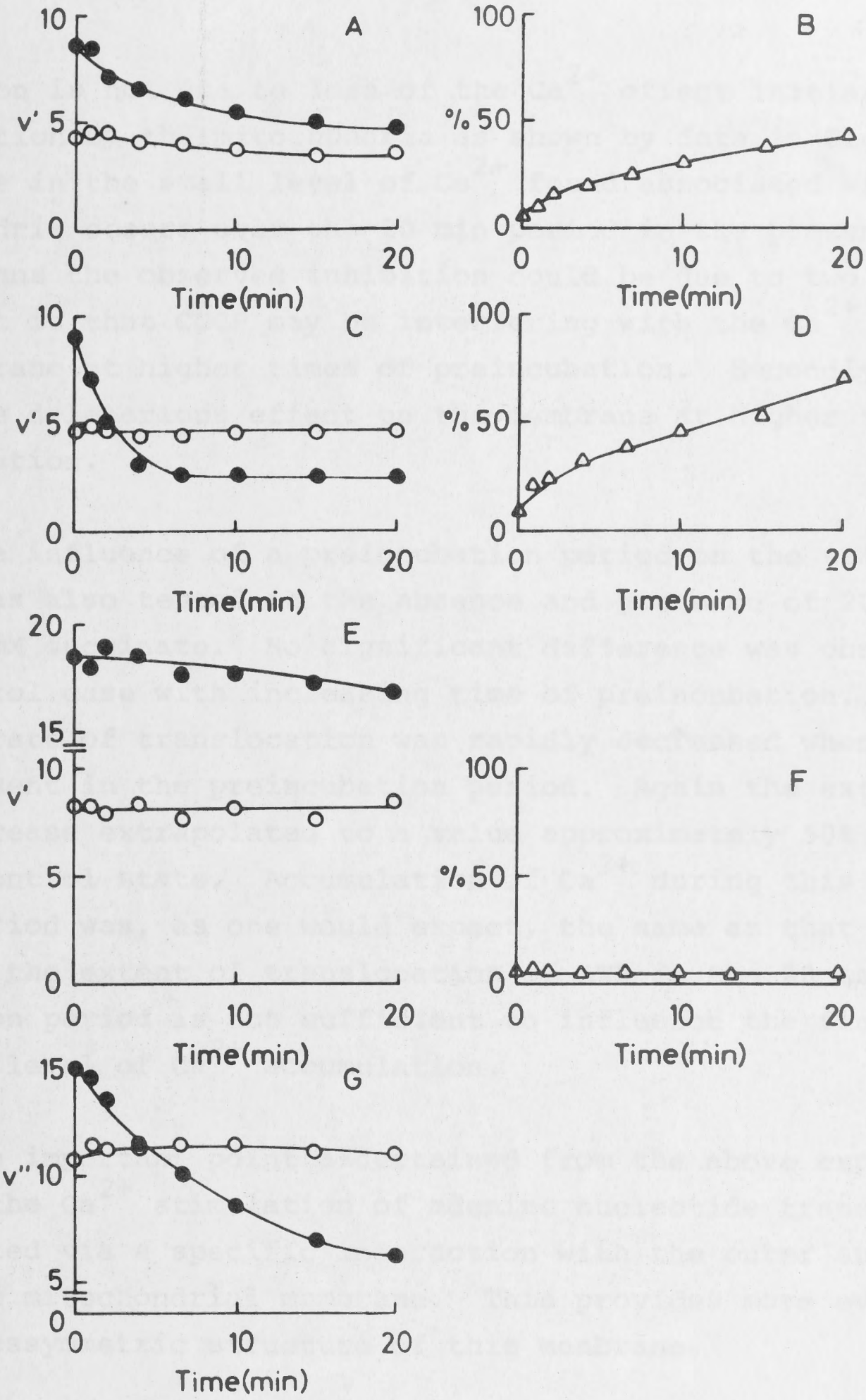


Figure 15. *Influence of preincubation time with Ca^{2+} on Ca^{2+}-stimulated adenine nucleotide translocation.*

Mitochondria were incubated essentially as in method (b) in a basic medium supplemented with 200μM ATP or ADP and 200μM Ca^{2+}, 200μM succinate and 5μM CCCP as indicated. Preincubation times with all ingredients, except adenine nucleotide, were from 0 to 20 minutes. Ca^{2+} accumulation by the mitochondria was determined as described in Section B. A, ATP; C, ATP plus succinate; E, ATP plus CCCP; G, ADP plus succinate. B, D and F refer to the Ca^{2+} accumulated in A, C and E respectively. ○ , Ca^{2+} absent; ● , Ca^{2+} present; v' and v" refer to the rate of ATP and ADP translocation, respectively, expressed in nmoles per min per mg protein; % refers to the % of added Ca^{2+} accumulated in the 20 second incubation period by the mitochondria.

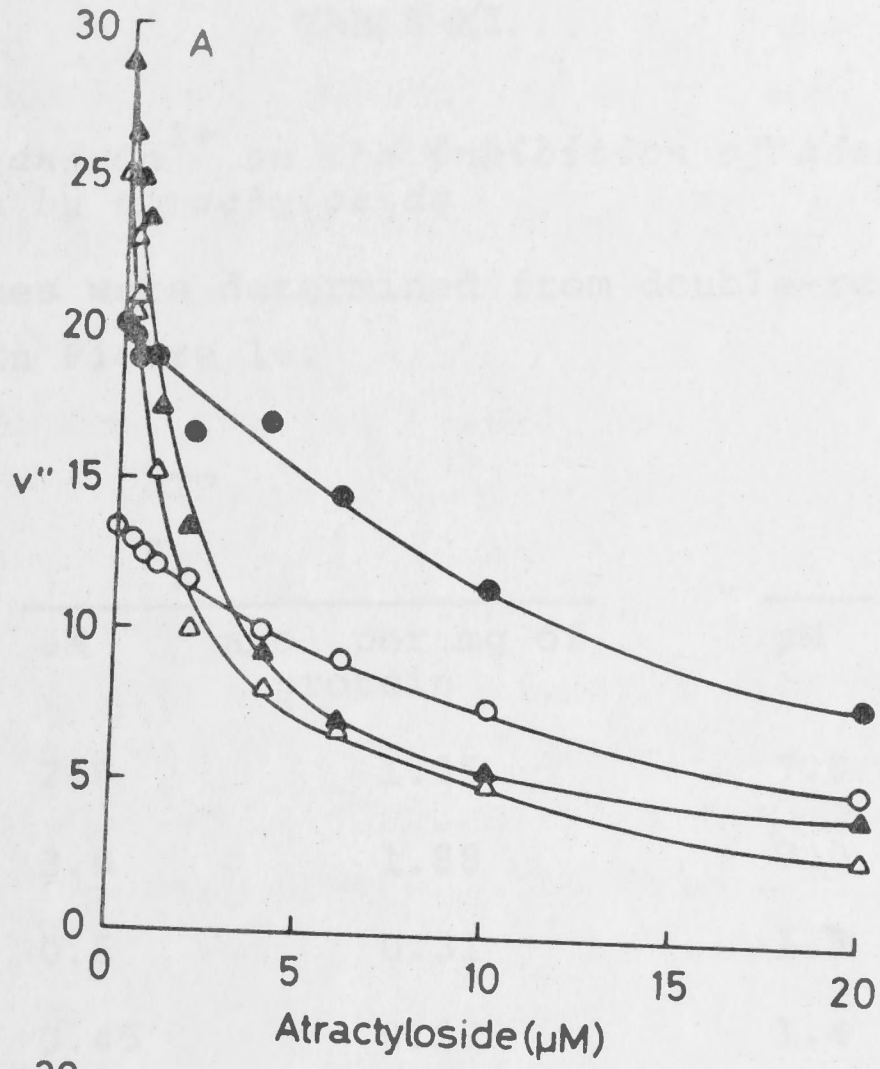
inhibition is not due to loss of the Ca^{2+} effect initiated by its accumulation by the mitochondria as shown by data in Figure 15f. No change in the small level of Ca^{2+} found associated with the mitochondria occurs over the 20 min period in the presence of CCCP. Thus the observed inhibition could be due to two factors. The first is that CCCP may be interfering with the Ca^{2+} effect on the membrane at higher times of preincubation. Secondly, Ca^{2+} may have some deleterious effect on the membrane at higher times of preincubation.

The influence of a preincubation period on the translocation of ADP was also tested in the absence and presence of 200μM Ca^{2+} plus 0·2mM succinate. No significant difference was observed in the control case with increasing time of preincubation. As with ATP the rate of translocation was rapidly decreased when Ca^{2+} was also present in the preincubation period. Again the extent of this decrease extrapolated to a value approximately 50% lower than in the control state. Accumulation of Ca^{2+} during this preincubation period was, as one would expect, the same as that observed for ATP; the extent of translocation of ATP in the 20 second incubation period is not sufficient to influence the final observed level of Ca^{2+} accumulation.

The important point ascertained from the above experiments is that the Ca^{2+} stimulation of adenine nucleotide translocation is mediated via a specific interaction with the outer surface of the inner mitochondrial membrane. This provides more evidence for the assymmetric structure of this membrane.

Influence of K^+ and Ca^{2+} on the affinity of the mitochondrial membrane for atractyloside

As Meisner (165) has pointed out one may distinguish between cations influencing the binding of adenine nucleotide to the membrane by using atractyloside as a probe. This inhibitor of the translocation reaction is a trivalent anion at neutral pH which is bound on the outer surface of the inner membrane. Estimation of its binding to the membrane may be done by measuring either the change in the respiratory control ratio, or the effect on the translocation of adenine nucleotide across the membrane, the latter being a more direct means. Data in Figure 16 shows the effect of increasing atractyloside concentration on the inhibition of ATP



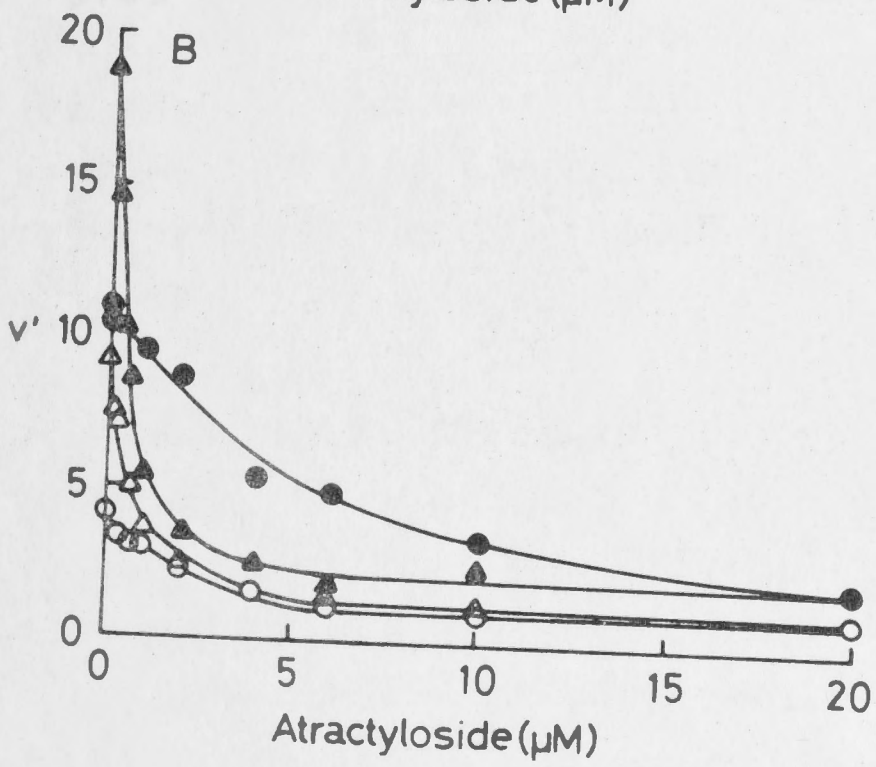


Figure 16. *Influence of K^+ and Ca^{2+} on the inhibition of adenine nucleotide translocation by atractyloside.*

Mitochondria were incubated by method (b) in a basic medium supplemented with 200μM ATP or ADP, various concentrations of atractyloside and 20mM K^+ and 200μM Ca^{2+} as indicated. A, ADP; B, ATP; ○ , no addition; ● , Ca^{2+}; △ ,K^+; ▲ , Ca^{2+} plus K^+. v' and v" refer to the rate of translocation of ATP and ADP, respectively, in nmoles/min per mg protein.

TABLE XI

Effect of K^+ and Ca^{2+} on the inhibition of adenine nucleotide translocation by atractyloside

K_i values were determined from double-reciprocal plots of the data in Figure 16.

	ATP		ADP	
	K_i		K_i	
Additions	μM	nmol per mg of protein	μM	nmol per mg of protein
None	2.8	1.25	7.0	4.35
Ca^{2+}	3.0	1.88	7.0	4.35
KCl	0.5	0.31	1.5	0.94
Ca^{2+} + KCl	0.45	0.29	1.4	0.87

and ADP translocation in the presence and absence of K^+ and/or Ca^{2+}. Inhibitor constants obtained from these plots are tabulated in Table XI both expressed as a function of μM added and nmoles per mg of mitochondrial protein. The following pieces of information are worth noting. Firstly, as one would expect for a competitive inhibitor, the values of the K_i for ATP translocation are lower than those for the translocation of ADP under corresponding conditions. Secondly, K^+ increases the affinity of the translocase for atractyloside. This increase in affinity for atractyloside is large as reflected in the decrease in the K_i, in the case of ATP, from 2·8 to 0·5μM, approximately six-fold. These results imply that the binding of the adenine nucleotide, rather than its actual transport through the mitochondrial inner membrane is altered by K^+ ions. However, the inhibitor constants in the presence of Ca^{2+} are approximately the same as those obtained in the absence of Ca^{2+}. The fact that the K_i values are similar in the absence and presence of Ca^{2+} may be an artificial situation brought about by (a) the increase of translocation in the presence of Ca^{2+}, which would increase the K_i, and (b) an actual increase in the affinity caused by the Ca^{2+} binding to the membrane, which would decrease the K_i.

This hypothesis may be clarified somewhat by the use of carboxyatractyloside, an analogue of atractyloside possessing four net negative charges at neutral pH (Figure 17). By analogy with ATP, which also has four net negative charges one would expect differences in the absence and presence of Ca^{2+} with respect to the binding/affinity of this inhibitor. There is a decrease in the value of K_i observed on the addition of Ca^{2+} both for ATP and ADP for carboxyatractyloside. This decrease is larger in the case of ADP (K_i falls 50%) than ATP (K_i falls 15%) Table (XII). One would be tempted to say from this observation that the Ca^{2+} ions act by increasing the binding of the adenine nucelotide to the mitochondria. This conclusion is, however, in contrast to experiments reported by Weidemann *et al* (267) on the direct measurement of ADP binding to mitochondrial membranes. These workers found that, if anything, Ca^{2+} ions decreased the extent of this binding.

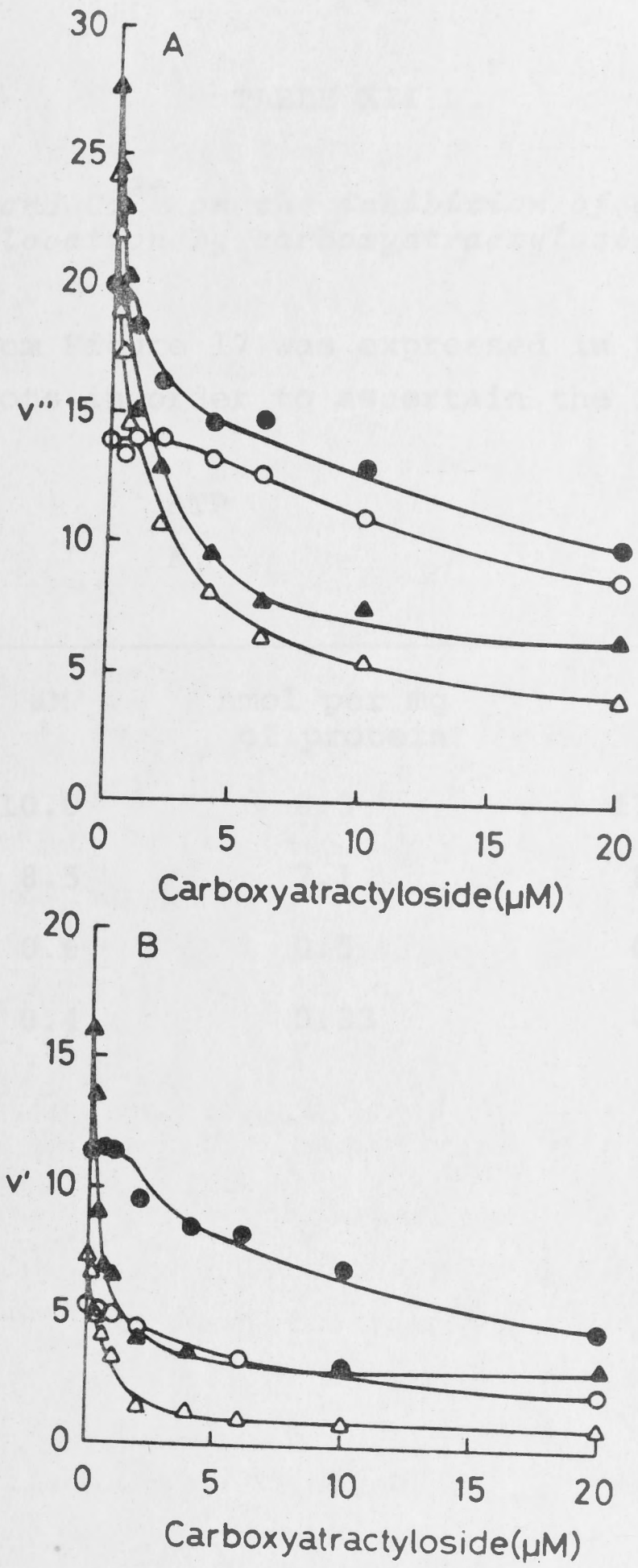


Figure 17. *Influence of K^+ and Ca^{2+} on the inhibition of adenine nucleotide translocation by carboxyatractyloside.*

Conditions were as indicated in the legend to Figure 16 except that the carboxyatractyloside concentration was varied. A, ADP; B, ATP; ○, no addition; ●, Ca^{2+}; △, K^+; ▲, Ca^{2+} plus K^+. V' and V" refer to the rate of translocation of ATP and ADP, respectively, in nmoles/min per mg protein.

TABLE XII

Effect of K^+ and Ca^{2+} on the inhibition of adenine nucleotide translocation by carboxyatractyloside

Data from Figure 17 was expressed in terms of double reciprocal plots in order to ascertain the inhibitor constants.

	ATP		ADP	
	K_i		K_i	
Additions	µM	nmol per mg of protein	µM	nmol per mg of protein
None	10.0	8.3	17.0	14.2
Ca^{2+}	8.5	7.1	8.5	7.1
KCl	0.6	0.5	0.9	0.75
Ca^{2+} + KCl	0.4	0.33	0.6	0.5

Several differences are evident between the inhibitory effects of atractyloside and carboxyatractyloside. Firstly, there is a greater relative increase in the K_i in the presence of K^+ for the latter. This is due not to an actual greater affinity for the inhibitor in the presence of K^+, but to a decreased affinity in the absence of K^+ caused by the extra net negative charge on the carboxyatractyloside inhibiting its binding to the predominantly negative mitochondrial membrane. Secondly, in the absence of K^+ the inhibition by carboxyatractyloside is sigmoidal whilst in its presence the inhibition is hyperbolic. Ca^{2+} has no effect on this property. Vignais *et al* (260) have observed cooperative binding of carboxyatractyloside to the mitochondrial membrane which would explain this but from their results this cooperativity was not lost in the presence of K^+ ions. It is possible that the binding remains sigmoidal in the presence of K^+ but that the interaction with the translocase, resulting in the inhibition, is modified in some way to sensitize the translocase reaction to the presence of carboxyatractyloside.

Influence of preincubation of mitochondria with atractyloside on the loss of endogenous adenine nucleotides

Early experiments indicated that the amount of endogenous adenine nucleotide lost from the mitochondria increased in the presence of Ca^{2+}. In an effort to investigate this phenomenon it was decided to see whether this value could be lowered by preincubating the mitochondria with atractyloside. As shown in Figure 18 no change was observed in the leakage of endogenous adenine nucleotide in the absence of added ATP. One point worth noting is that the addition of Ca^{2+} increased the leakage, perhaps due to either a displacement of adenine nucleotide bound at sites on the membrane or to a slight reversible increase in permeability of the membrane. The most interesting results were obtained when ATP was also present in the incubation medium. Under these conditions it is found that, using a 60 second preincubation period of the mitochondria with atractyloside, a 25% loss of endogenous adenine nucleotide was observed in the absence and 50% in the presence of 200μM Ca^{2+}. In the short time and under the conditions used one would not expect the added atractyloside to be inactivated in any way by the mitochondria. In spite of this

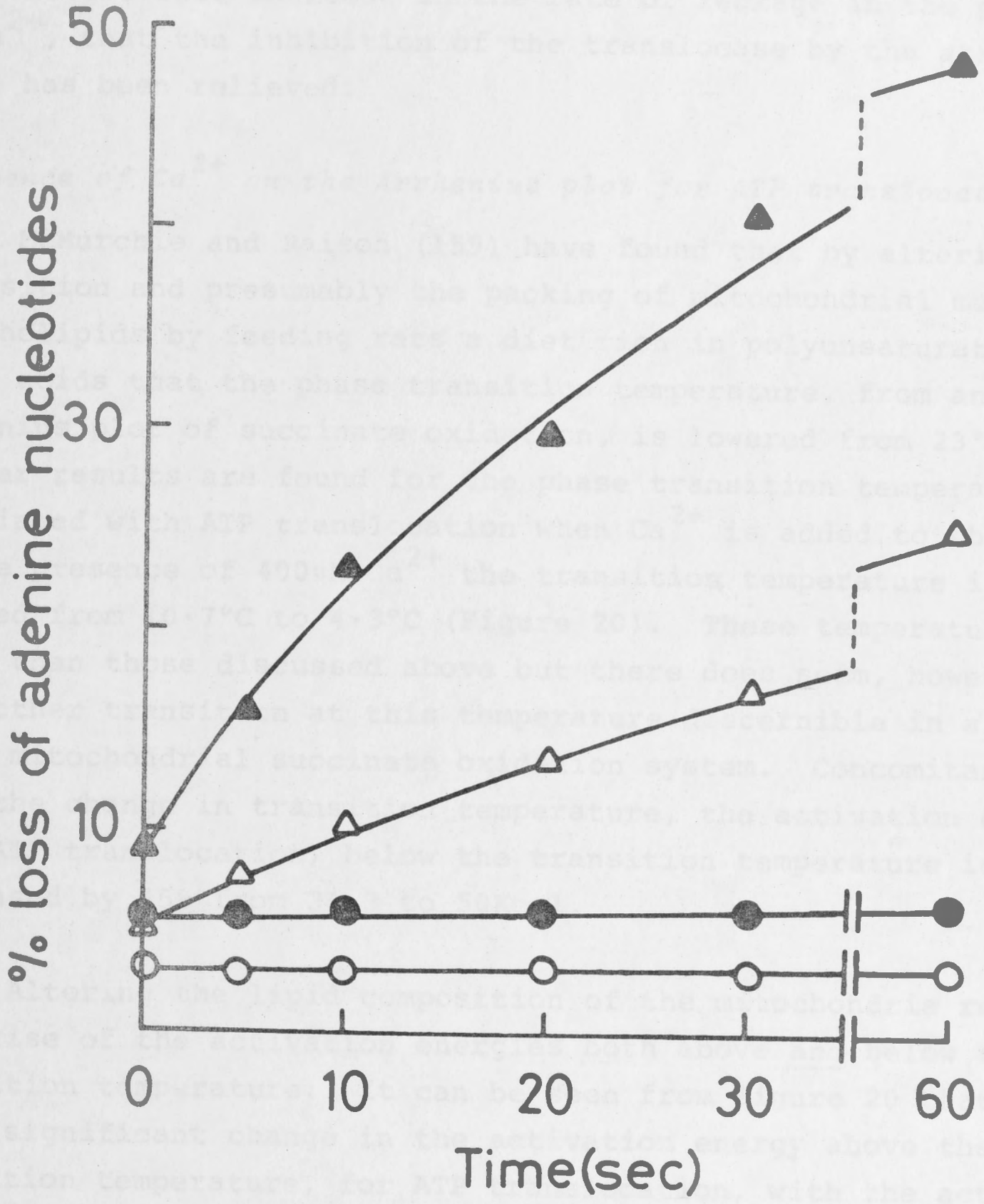


Figure 18. *Influence of preincubation of mitochondria with atractyloside on the loss of endogenous adenine nucleotide.*

Mitochondria were incubated essentially by method (b) in a basic medium supplemented with 200μM Ca^{2+} and 200μM ATP as indicated. Mitochondria were preincubated with 50μM atractyloside for the times indicated and the reaction terminated after a 20 second incubation period by separation of the mitochondria by centrifugation. ○ , no addition; ● , Ca^{2+}; ▲ , Ca^{2+} plus ATP; △ ,ATP.

it is obvious from the results obtained in the presence of ATP, i.e. the two-fold increase in the rate of leakage in the presence of Ca^{2+}, that the inhibition of the translocase by the atractyloside has been relieved.

Influence of Ca^{2+} on the Arrhenius plot for ATP translocation

McMurchie and Raison (159) have found that by altering the composition and presumably the packing of mitochondrial membrane phospholipids by feeding rats a diet rich in polyunsaturated fatty acids that the phase transition temperature, from an Arrhenius plot of succinate oxidation, is lowered from 23°C to 5°. Similar results are found for the phase transition temperature associated with ATP translocation when Ca^{2+} is added to the system. In the presence of 400µM Ca^{2+} the transition temperature is lowered from 10·7°C to 4·3°C (Figure 20). These temperatures are lower than those discussed above but there does seem, however, to be another transition at this temperature discernible in a sheep liver mitochondrial succinate oxidation system. Concomitant with the change in transition temperature, the activation energy (for ATP translocation) below the transition temperature is increased by 45% from 34·3 to 50Kcal.

Altering the lipid composition of the mitochondria resulted in a rise of the activation energies both above and below the transition temperature. It can be seen from Figure 20 that there is no significant change in the activation energy above the transition temperature, for ATP translocation, with the activation energy staying constant at approximately 18·2Kcal.

The results presented here, especially the lowered transition temperature, are consistent with an increased mobility being 'imposed' on the membrane in the presence of Ca^{2+}. This increased mobility not only influences the properties of the membrane which determine the transition temperature, but also influence the hydrophobic, lipid-protein interactions in the membrane perhaps by neutralising the negative charges on the surface of the membrane. This results in an altered conformation of the membrane and the translocase as indicated by the change in activation energy.

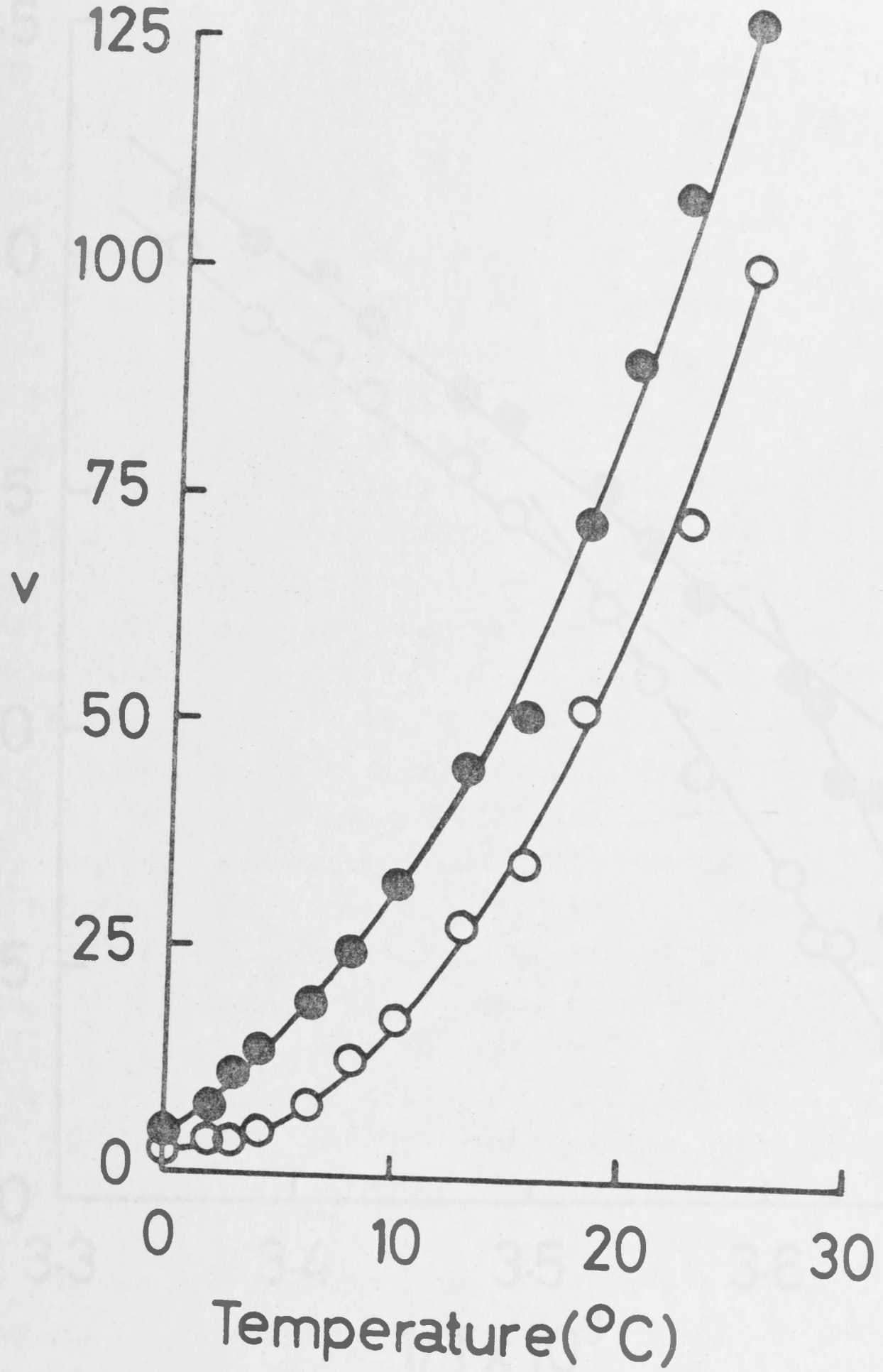


Figure 19. *Influence of temperature on Ca^{2+}-stimulated ATP translocation.*

Mitochondria were incubated as in method (b) using a one minute preincubation period in a basic medium supplemented with 400µM ATP and 400µM Ca^{2+} when indicated. The incubation times varied from 30 to 5 seconds and the temperatures tested from 0 to 25°C. ○ , no addition; ● , Ca^{2+}. V refers to the rate of translocation of ATP in nmoles/min per mg protein.

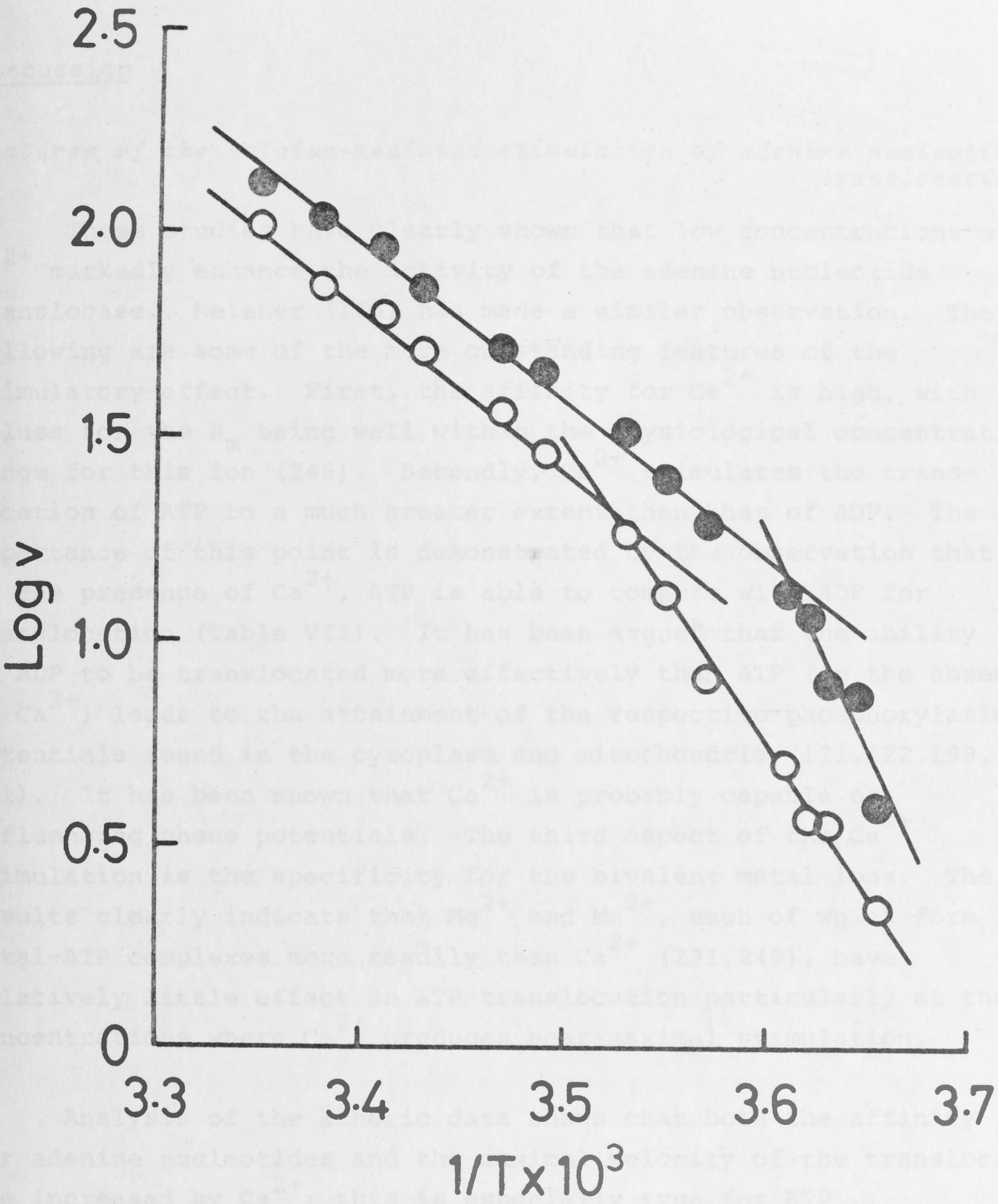


Figure 20. *Influence of Ca^{2+} on the Arrhenius plot for ATP translocation.*

Data from Figure 19 was recalculated in the form of Arrhenius suitable for the determination of activation energies and transition temperatures. ○ , no addition; ● , Ca^{2+}.

Discussion

Features of the calcium-mediated stimulation of adenine nucleotide translocation

These studies have clearly shown that low concentrations of Ca^{2+} markedly enhance the activity of the adenine nucleotide translocase. Meisner (165) has made a similar observation. The following are some of the more outstanding features of the stimulatory effect. First, the affinity for Ca^{2+} is high, with values for the K_m being well within the physiological concentration range for this ion (246). Secondly, Ca^{2+} stimulates the translocation of ATP to a much greater extent than that of ADP. The importance of this point is demonstrated by the observation that, in the presence of Ca^{2+}, ATP is able to compete with ADP for translocation (Table VII). It has been argued that the ability of ADP to be translocated more effectively than ATP (in the absence of Ca^{2+}) leads to the attainment of the respective phosphorylation potentials found in the cytoplasm and mitochondria (121,122,199, 201). It has been shown that Ca^{2+} is probably capable of influencing these potentials. The third aspect of the Ca^{2+} stimulation is the specificity for the bivalent metal ions. The results clearly indicate that Mg^{2+} and Mn^{2+}, each of which form metal-ATP complexes more readily than Ca^{2+} (231,245), have relatively little effect on ATP translocation particularly at those concentrations where Ca^{2+} produces near-maximal stimulation.

Analysis of the kinetic data shows that both the affinity for adenine nucleotides and the maximal velocity of the translocase are increased by Ca^{2+}; this is especially true for ATP (see Figures 5 and 6). This suggest that Ca^{2+} may be producing two effects on the translocase. First it could be stimulating the translocating by binding to the mitochondrial membrane in the vicinity of the translocase (see below). Secondly, the $CaATP^{2-}$ species, which as mentioned above seems to be a substrate for the translocase, may also be influencing the kinetic characteristics of the translocase. This would in part explain two observations. Firstly, the specificity of the stimulation for ATP; the stability constant for $CaADP^{-}$ is more than an order of magnitude less than that for $CaATP^{2-}$ (231,245). Secondly, the incomplete inhibition of Ca^{2+}-stimulated ATP translocation by La^{3+}, Nd^{3+} and Ruthenium Red; it would require a relatively large concentration of La^{3+} to inhibit the formation of $CaATP^{2-}$.

The very high affinity of Ca^{2+} stimulated ATP translocation for ATP, particularly in the presence of K^{+}, is noteworthy. The values obtained for affinity constants lie within the range observed for other parameters of oxidative phosphorylation (see 26,38). They are higher than the values reported by Pfaff *et al* (201) obtained from experiments with low concentrations of mitochondria. Presumably the values obtained in the present work would have been even lower if lower concentration of mitochondria had been used in the reaction mixtures. Such low concentrations of mitochondria are highly unrepresentative of the concentrations prevailing in the cell cytoplasm; rough estimates of this value place the concentrations in rat liver at more than 10mg of protein per ml.

Tentative mechanism by which Ca^{2+} stimulates adenine nucleotide translocation

The means by which Ca^{2+} stimulates the translocation of ATP cannot be elucidated solely from the data presented in this section. Some tentative conclusions, however, can be reached. It appears unlikely that accumulation of Ca^{2+} is obligatory for stimulation of ATP translocation. Information from experiments with the uncoupler CCCP indicates that Ca^{2+} itself is not acting as an uncoupler and thereby directly influencing the proton gradient across the inner mitochondrial membrane (cf.201). This is supported by the additive nature of the Ca^{2+} and uncoupler-stimulated exchanges (Table III). Indeed information gained from following the accumulation of Ca^{2+} during the translocation of ATP is convincing direct evidence for this hypothesis. Stimulation of ATP translocation by K^{+} was also shown to be additive with that of uncouplers (165) and this has been confirmed. Similarly, oligomycin, which inhibits ATP-supported Ca^{2+} accumulation, had little effect on Ca^{2+}-stimulated ATP translocation. Further, the bivalent metal ion specificity for stimulation of ATP translocation bears little relation to the specificity for accumulation of bivalent ions. The results of Carafoli (30) would suggest that the apparent order for accumulation by isolated rat liver mitochondria is $Ca^{2+}=Sr^{2+}>>Mn^{2+}=Ba^{2+}$. Mg^{2+} is not accumulated by these mitochondria to any large degree (147).

Rare earth cations such as La^{3+} and Nd^{3+} and the compound Ruthenium Red once more have illustrated the usefulness of such Ca^{2+} antagonists in elucidating the effects on biological processes. By the use of these compounds it has been shown that the response of ADP translocation is clearly different to that of ATP translocation. This was particularly evident when low concentrations of Ca^{2+} were simultaneously present in the incubation mixture. Secondly conclusive evidence is forthcoming from the use of these antagonists to indicate that the stimulation induced by Ca^{2+} is different from that induced by uncouplers and that induced by K^{+}. The observations that La^{3+} inhibits only that portion of ATP translocation which is stimulated by Ca^{2+} and competes with added Ca^{2+}, suggests a mechanism of stimulation which involves a binding site(s) for Ca^{2+} in the vicinity of the translocase and not one involving, for example, chelation of metal ion with ATP, although this latter option may still be possible. One interesting observation from the use of La^{3+} was that it did not produce the same inhibitory nor stimulatory effects for ATP and ADP, respectively, in the Mg^{2+}-stimulated system as compared to the Ca^{2+} -stimulated system. This together with the different affinities of Mg^{2+} for ATP and ADP translocation indicates that the Mg^{2+} stimulatory mechanism differs markedly from that for Ca^{2+}. Such a result may be unexpected in light of the fact that both are bivalent cations and that Ca^{2+} may substitute for Mg^{2+} in Mg^{2+} - requiring reactions. albeit with disastrous consequences as regards the activity of the reaction. Conversely Mg^{2+} has been shown to be a competitive inhibitor of Ca^{2+} binding to mitochondrial membranes (110,212). The point of difference must lie then not in the binding to the membrane but the conformational or other change induced in the membrane which results in the stimulation. Differences were also noticed between Ca^{2+} and Mg^{2+} on the basis of their stimulatory properties in the presence of K^{+} ions. Whereas K^{+} and Ca^{2+} potentiated the effects of each other, K^{+} was inhibitory in a Mg^{2+}-stimulated system. Although the effects produced by K^{+} and Ca^{2+} were separable under some conditions, such as above, there was some degree of similarity present. This is probably related to the cationic nature of both of these agents. In relation to this it has been observed that K^{+}, like Mg^{2+} is a competitive inhibitor of Ca^{2+} binding to mitochondrial membranes (212,215).

Four independent pieces of information raise the possibility that the enhanced translocase activity results from an interaction of Ca^{2+} with phospholipids located in the environment of the translocase. First the affinity of the Ca^{2+}-stimulated translocation for Ca^{2+} is of the order found for the mitochondrial low affinity for Ca^{2+} binding sites (32,210,212); these are probably lipid in nature (212,213). Interaction with phospholipids is also consistent with the bivalent ion specificity for K^{+} self-diffusion across phosphatidylsenine bilayers (189). Thirdly, valinomycin, which is thought to interact with membrane phospholipids (see for example 205), prevents to a considerable extent the ability of Ca^{2+} to stimulate ATP translocation (Table IV). The other point is that U^{2+} ions which are known to bind to the phosphate moiety of phospholipids (227), although they inhibit gross translocation of adenine nucleotide, show a preference in this inhibition for the Ca^{2+}-stimulated portion (see insert to Figure 4). In relation to the phospholipid involvement Kreutz (133) has indicated that Ca^{2+} ions are able to increase the permeability of artificial phospholipid membranes.

Other data concerning the site of Ca^{2+} binding on the membrane may be gleaned from an investigation of the chemistry of Ca^{2+} ions (see ref. 80). The partial effectiveness of Mg^{2+} in mimicking Ca^{2+} reflects the fact that the lower ionic potential of the Ca^{2+} moiety is favoured. The lack of a requirement for eight co-ordination is shown by the observation that Ba^{2+}, which is even more suited to this co-ordination number than Ca^{2+}, is barely effective. Another, and probably the most important property of Ca^{2+}, is its high affinity for oxygen ligands; the binding sites on phospholipid consist just of such ligands.

In contrast to Ca^{2+} and Mg^{2+} which bind to mitochondrial lipids, Zn^{2+} and other heavy metals (except U^{2+}) have been shown to bind to the protein moiety of the mitochondrial membrane (34, 35,36,107,110,222). This binding has also been shown to involve thiol groups on these proteins and so it is quite understandable that these compounds invoke leakage and lysis of the membranes. Of particular interest with respect to the involvement of the thiol groups is the observation that the binding of ADP (or ATP) to the translocase unmasks some membrane SH groups by inducing conformational changes in the membrane (139,258). This type of effect

one would perhaps expect to be mimicked by atractyloside. This then may be one way of explaining the increased lability of Zn^{2+} ions on the mitochondrial membrane in the presence of atractyloside. The effect of atractyloside on the heavy metal ion-induced leakage of adenine nucleotide from the mitochondrion as well as the observation that preincubation with atractyloside may also induce leakage under certain conditions emphasises how little we really know concerning the interaction of this inhibitor both with the translocase and with the mitochondrial membrane in general.

The observation that Ca^{2+}-stimulation of adenine nucleotide translocation is inhibited when the Ca^{2+} has been at least partly accumulated within the mitochondrion raises several possibilities: (a) there are no Ca^{2+} binding sites *per se* on the inner side of the inner membrane. Lehninger, however, has indicated that 'opening' of the mitochondria by valinomycin results in an increase in Ca^{2+} binding ability of the mitochondria due presumably to exposure of sites on the inside of the membrane (148). (b) there are binding sites but they do not induce a similar stimulation of adenine nucleotide translocation and, (c) that Ca^{2+} which enters the mitochondrion is preferentially bound at other protein and/or lipid sites. Furthermore the decreased level of translocation observed in the presence of an energy source (see Figure 15), as mentioned above cannot be accounted for on the basis of Ca^{2+} accumulated. Energisation of the mitochondrion is known to induce gross conformational changes in the membrane structure (82,84,266). Perhaps under these conditions conformational changes are also imparted to the translocase which leads to the inhibition. This effect could not be due solely to energisation as it is not seen in the absence of Ca^{2+}. This along with the fact that no inhibition is seen in the presence of CCCP implies that the Ca^{2+} must be on the outside of the mitochondrion to exert its effects.

Significance of Ca^{2+}-stimulated adenine nucleotide translocation with regard to cellular metabolism

Any enzyme or enzyme system that functionally links one cell compartment with another must play a central role in cell metabolism. In this repsect the adenine nucleotide translocase

is especially important since it performs two interrelated functions. First, it is able to control the relative concentrations of ATP and ADP, perhaps the important chemical species in the cell, in the cytoplasm and mitochondrial matrix. These two compounds are involved in many aspects of cell metabolism, if not as substrates for a reaction then certainly as chemical effectors or modifiers of numerous enzymic reactions. Secondly, the translocase is responsible for the transport of ADP and ATP into and out of the mitochondria. In this way it acts as a channel in the energy metabolism of the cell by 'assisting' in the production of ATP, the universal energy donor. Thus any physiological substance that can alter the activity of this and other controlling enzyme systems must, in itself, be involved in the control of cell metabolism.

The present findings suggest that Ca^{2+} stimulation of the adenine nucleotide translocase may have a physiological role in the cell. Figure 21 shows a proposed 'feed-forward' mechanism whereby the increased ATP translocated in the presence of Ca^{2+} would be available for hydrolysis by the adenosine triphosphatase (ATPase). The energy produced during ATP breakdown would enable Ca^{2+} to be accumulated via the Ca^{2+} permease. Thus the accumulation of Ca^{2+} by mitochondria would be controlled to a certain extent by its own concentration outside the mitochondria. Consistent with this argument is the finding that the accumulation of Ca^{2+} by rat liver mitochondria as a function of Ca^{2+} concentration follows sigmoidal saturation kinetics (27,28,236,261).

One question to be asked is 'What is the significance of the entry of the Ca^{2+} into the mitochondrion'? The answer to this is not simple but may be related to the role of Ca^{2+} as an inhibitor/activator of a variety of metabolic reactions. An obvious example of this occurs in relation to muscle where it has been suggested that in addition to the cytoplasmic reticulum, the mitochondria also play a part in regulating calcium movements and contractile events. Swift removal of Ca^{2+} from the cytoplasm would be necessary for the relatively frequent contraction cycles. Evidence for the involvemement of heart mitochondria in contraction of this particular tissue has come from various sources (33,87). One piece of evidence which rules out the involvement of mitochondria is the kinetics of Ca^{2+} accumulation

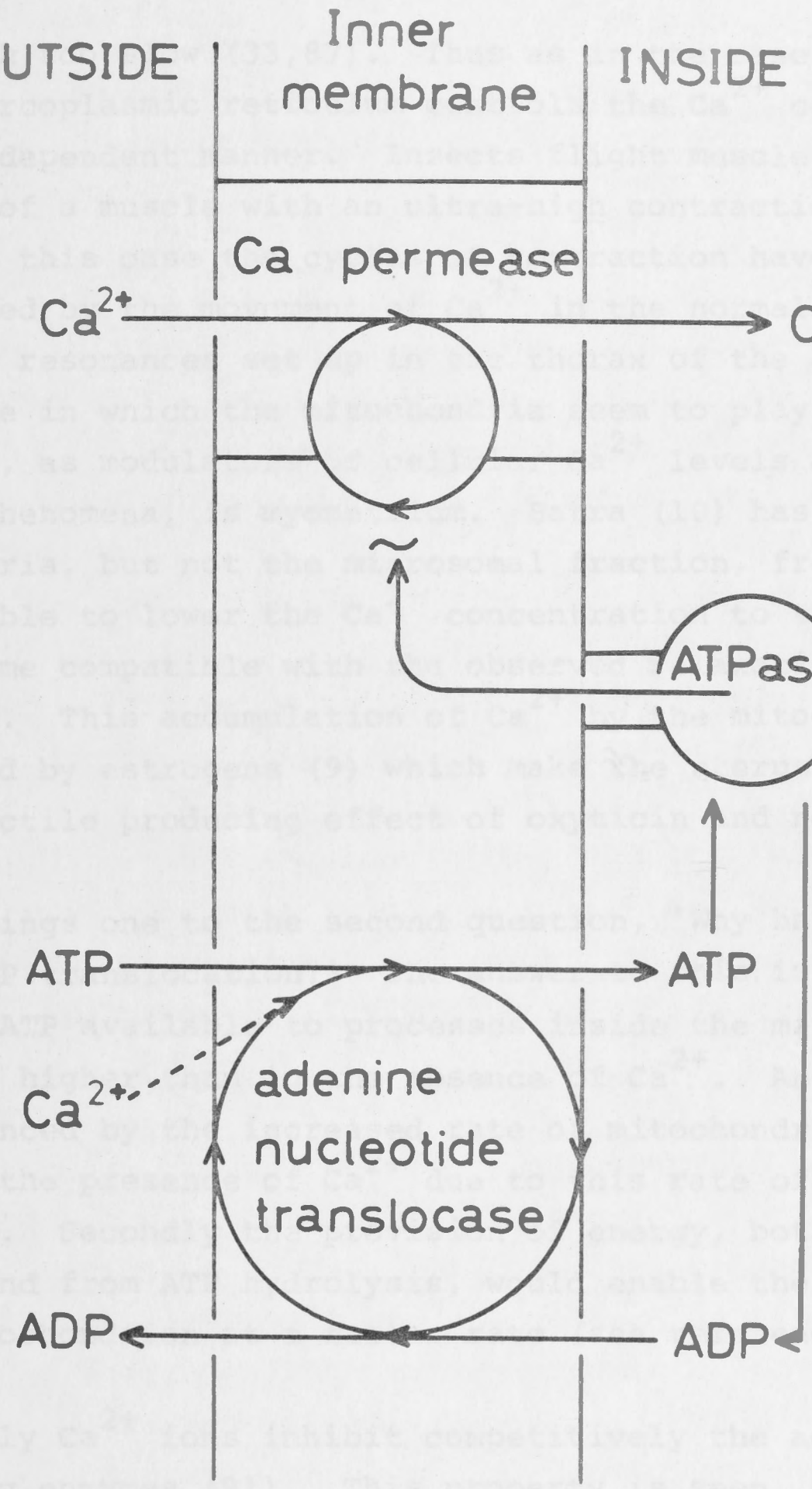


Figure 21. *Envisaged link through Ca^{2+} between Ca^{2+} permease and adenine nucleotide translocase.*

The dashed line indicates an interaction of Ca^{2+} with the translocase. This interaction does not necessarily involve concomitant translocation of Ca^{2+} with the ATP. ~ is the energy pressure necessary for Ca^{2+} accumulation.

which are much too slow (33,87). Thus as in the case of skeletal muscle the sarcoplasmic reticulum controls the Ca^{2+} concentration in an energy-dependent manner. Insects flight muscle is an extreme case of a muscle with an ultra-high contraction/relaxation frequency; in this case the cycles of contraction have been shown to be initiated by the movement of Ca^{2+} in the normal way but maintained by resonances set up in the thorax of the animal. A type of muscle in which the mitochondria seem to play the dominant role, as modulators of cellular Ca^{2+} levels and consequent contraction phenomena, is myometrium. Batra (10) has shown that the mitochondria, but not the microsomal fraction, from this tissue were able to lower the Ca^{2+} concentration to the threshold level in a time compatible with the observed relaxation in this smooth muscle. This accumulation of Ca^{2+} by the mitochondria is also inhibited by estrogens (9) which make the uterus more amenable to the contractile producing effect of oxyticin and norepinephrine.

This brings one to the second question, 'Why have Ca^{2+}-stimulated ATP translocation?' The answer to this is twofold; firstly, the ATP available to processes inside the mitochondrion would be much higher than in the absence of Ca^{2+}. An exmaple of this is evidenced by the increased rate of mitochondrial protein synthesis in the presence of Ca^{2+} due to this rate of ATP translocation (12). Secondly the provision of energy, both from respiration and from ATP hydrolysis, would enable the Ca^{2+} to enter the mitochondrion at a faster rate (see reference 236).

Generally Ca^{2+} ions inhibit competitively the activity of Mg^{2+}-requiring enzymes (91). This property is seen, for example, in the glycolytic enzyme pyruvate kinase which has an obligatory requirement for Mg^{2+} (25,116,172) and a monovalent cation, usually K^{+} (19). There also exists enzymes such as malic enzyme from adrenal cortex mitochondria which has a mandatory requirement for both Mg^{2+} and Ca^{2+} (202).

It is possible to envisage that under certain circumstances the Mg^{2+}/Ca^{2+} ratio could become a physiological factor that is capable of influencing the absolute rate of a single enzyme or metabolic pathway sensitive to this ratio. If cells then had the

capacity to alter this ratio in a specific environment, be it cytoplasmic or mitochondrial, they would have at their disposal a further means of controlling or, at the very least, modifying metabolism.

Such a situation has been shown to arise using an *in vitro* system of pyruvate kinase and rat liver mitochondria (170). Mitochondria from these cells are known to accumulate Ca^{2+} but not Mg^{2+} (167) and thus it is a simple exercise to alter the Mg^{2+}/Ca^{2+} ratio and consequently the activity of the pyruvate kinase under these conditions.

The involvement of Ca^{2+} and Mg^{2+} in pyruvate metabolism may be extended further by introducing two other enzymes, pyruvate carboxylase and pyruvate dehydrogenase Figure 22 shows the interrelationships between pyruvate and the above three enzymes with regard to their localisation in the cell and known effector agents. From such a scheme it is possible to envisage a situation(s) where Ca^{2+} and ATP, by way of their respective transport into the mitochondria, may be able to affect the overall flux through these systems.

With regard to the activity of the respective enzymes the following factors may have to be taken into account: (a) pyruvate carboxylase is inhibited by Ca^{2+} (117) and stimulated by acetyl CoA (250). Thus when the mitochondrion has a high level of energy in the form of both high energy phosphate bonds (ATP) and oxidizable substrate (acetyl CoA) this, the first step in the production of glucose (gluconeogenesis) from pyruvate, is stimulated (b) pyruvate dehydrogenase is inhibited by ATP (152, 153) and activated by low levels of Ca^{2+}(57,197).[Mg^{2+} is also required for both the activation and inactivation steps (152,153)]. Higher concentrations of Ca^{2+} would be expected to inhibit the production of both active and inactive forms of this enzyme. (c) pyruvate kinase is inhibited by Ca^{2+} (25,114,172).

If, for example, one takes the hypothetical case where both ATP and Ca^{2+} concentrations are high in the cytoplasm then the immediate result is the pyruvate kinase and consequently the energy flow through the mitochondrion is inhibited. If this ATP were then to be translocated into the mitochondrion the subsequent

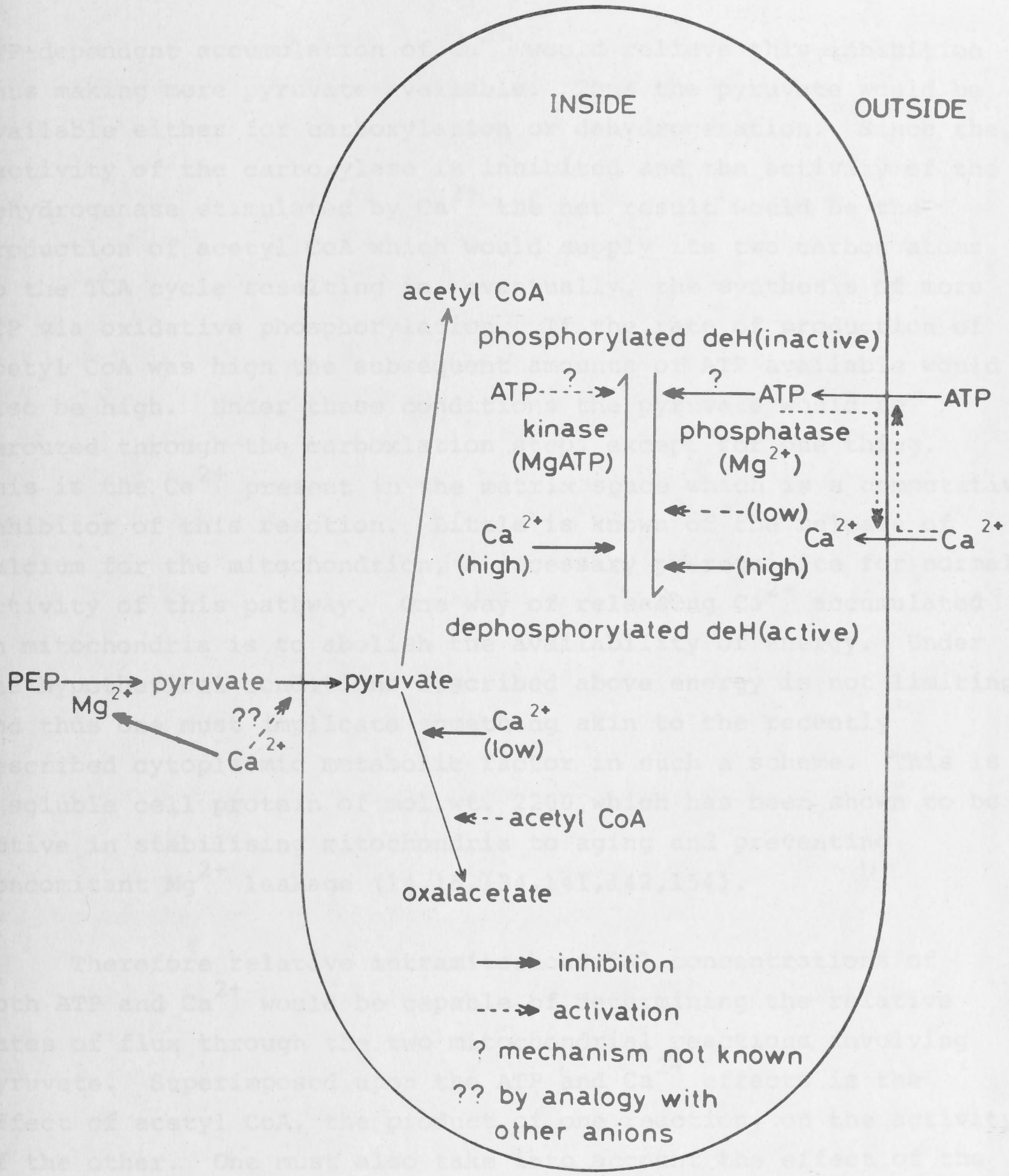


Figure 22. *Links between pyruvate metabolism in the mitochondrion, Ca^{2+} and ATP.*

ATP-dependent accumulation of Ca^{2+} would relieve this inhibition thus making more pyruvate available. Thus the pyruvate would be available either for carboxylation or dehydrogenation. Since the activity of the carboxylase is inhibited and the activity of the dehydrogenase stimulated by Ca^{2+} the net result would be the production of acetyl CoA which would supply its two carbon atoms to the TCA cycle resulting in, eventually, the synthesis of more ATP via oxidative phosphorylation. If the rate of production of acetyl CoA was high the subsequent amounts of ATP available would also be high. Under these conditions the pyruvate would be rerouted through the carboxlation step, except for one thing. This is the Ca^{2+} present in the matrix space which is a competitive inhibitor of this reaction. Little is known of the release of calcium for the mitochondrion, a necessary prerequisite for normal activity of this pathway. One way of releasing Ca^{2+} accumulated in mitochondria is to abolish the availability of energy. Under the hypothetical conditions described above energy is not limiting and thus one must implicate something akin to the recently described cytoplasmic metabolic factor in such a scheme. This is a soluble cell protein of mol.wt. 2200 which has been shown to be active in stabilising mitochondria to aging and preventing concomitant Mg^{2+} leakage (14,15,134,141,142,154).

Therefore relative intramitochondrial concentrations of both ATP and Ca^{2+} would be capable of determining the relative rates of flux through the two mitochondrial reactions involving pyruvate. Superimposed upon the ATP and Ca^{2+} effects is the effect of acetyl CoA, the product of one reaction, on the activity of the other. One must also take into account the effect of the extramitochondrial concentrations of Ca^{2+} principally on the initial availability of pyruvate from glycolysis. The issue is further complicated by the observation that Ca^{2+} ions stimulate the entry of di- and tricarboxylic acid metabolites of the TCA cycle into the mitochondrion (166). Furthermore interactions between other metabolites have also been described in the literature. These include: (a) an observed ADP requirement in the accumulation of Ca^{2+} and Pi by mitochondria (138), (b) PEP inhibition of Ca^{2+} accumulation by way of increasing the rate of efflux of Ca^{2+} (44); this effect is antagonised by ATP and is linked to the ability of the mitochondria to oxidize NADH-linked

substrates, (c) inhibition of ATP translocation by succinate and to a lesser extent pyruvate (42), (d) requirement for the Pi transporter to be operative during ADP translocation (158).

Another factor to be considered is the role played by the endoplasmic reticulum, which are also capable of sequestering Ca^{2+}, and the plasma membrane which is capable of transporting extracellular Ca^{2+} into the cell under for example hormone-mediated cyclic AMP directives. Obviously the number of permutations and combinations of substrate and effector concentrations that are capable of existence both extra- and intramitochondrially, and not only of Ca^{2+} and ATP, is vast. Comprehension of such a situation, even to a relatively small extent, is impossible within the realms of the human mind due to the large number of variables but perhaps may be simulated, in the not too distant future, by the use of computers.

Unlike DNA and RNA replication for example it is difficult to conceive of a situation in the cell where the flow of metabolites through a particular reaction sequence is completely stopped (except when the enzyme(s) itself is absent). What probably occurs is that this flow of metabolites is modulated and control exerted by subtle changes in the direction of the flow.

One would not expect the Ca^{2+}-stimulated ATP translocation to have a marked effect on the extramitochondrial phosphorylation potential due to the large reservoir of ATP in this compartment under steady-state conditions. However, one could conceive of a situation where the phosphorylation potential within the mitochondrion itself is increased. This would be a transient situation leading to increased Ca^{2+} accumulation supported by ATPase activity within a short time period, as indicated in Figure 21, and a consequent lowering.

SUMMARY

1 Added Ca^{2+} stimulates the translocation of ATP by isolated rat liver mitochondria.

2 The apparent K_m for added Ca^{2+} in stimulating the translocation of 200μM ATP is approximately 160μM (75μM 'free' Ca^{2+}).

3 The greatest stimulation of ATP translocation by Ca^{2+} occurs at the lower concentrations of ATP.

4 Sr^{2+} (and to a lesser extent Ba^{2+}) can replace Ca^{2+} whereas Mg^{2+} and Mn^{2+} have only little ability to stimulate ATP translocation.

5 U^{2+} ions inhibit overall adenine nucleotide translocation with preferential inhibition of the Ca^{2+}-stimulated portion being observed.

6 Heavy metal ions, e.g. Zn^{2+} and Cu^{2+}, induce leakage of adenine nucleotides from the mitochondria which may partially be reversed by the higher metal ion concentration or the presence of ATP and potentiated by the addition of atractyloside.

7 Translocation of dATP is also stimulated by Ca^{2+} whereas that of ADP is stimulated to only a small degree.

8 Studies with metabolic inhibitors and uncouplers provide evidence that stimulation by Ca^{2+} and uncouplers is additive and that the mechanism of Ca^{2+} stimulation does not seem to involve the high energy intermediate of oxidative phosphorylation.

9 In the presence of Ca^{2+} ATP is able to effectively compete with ADP for translocation.

10 Added K^+ further enhances the ability of Ca^{2+} to stimulate ATP translocation.

11 Added K^+ inhibits Mg^{2+}-stimulated translocation of both ADP and ATP.

12 La^{3+} at relatively low concentrations inhibits Ca^{2+}-stimulated translocation of 200μM ATP (K_i = 3·5μM) and potentiates the similar stimulation of ADP translocation (K_a = 8μM). Similar results were obtained with ruthenium red and Nd^{3+} two other Ca^{2+} antagonists.

13 La^{3+} does not affect either the K^+-stimulated or uncoupler-stimulated translocation of ATP thereby providing more evidence for different sites of action of these effectors.

14 La^{3+} inhibits Mg^{2+}-stimulated ADP translocation but has little effect on that of ATP.

15 K^+ ions markedly increase the affinity of adenine nucleotide translocation to inhibition by both atractyloside and carboxyatractyloside. In the case of the latter Ca^{2+} also decreases the K_i but to a much smaller extent.

16 Mitochondria which have accumulated Ca^{2+} from the suspending medium lose their ability to stimulate ATP translocation by Ca^{2+} thereby indicating that the Ca^{2+} interaction is specific for the outside of the inner membrane.

17 Preincubation of mitochondria with atractyloside before the addition of adenine nucleotide induces a leakage of endogenous adenine nucleotide.

18 The temperature break determined from Arrhenius plots for the translocation of ATP was decreased from 10·7 to 4·3°C in the presence of 400μM Ca^{2+}. Concomitantly the activation energy below the transition temperature increased from 34.3 to 50Kcal; that above the transition temperature remained unchanged.

19 These findings are discussed in relation to the mechanism of Ca^{2+}-stimulation and the potential involvement of Ca^{2+} in modifying reactions involved in the regulation of cell metabolism.

SECTION D MEMBRANE-ACTIVE AGENTS AND ADENINE NUCLEOTIDE TRANSLOCATION

Introduction

In the previous section the results from a kinetic investigation of Ca^{2+}-stimulated adenine nucleotide translocation were described which led to the proposition that mitochondrial phospholipids are intimately involved in the stimulatory process. As mentioned earlier the activity of any membrane-bound enzyme is likely to be dependent upon changes in the structure and/or conformation of the membrane itself and its components. This might be the case particularly for an enzyme such as the adenine nucleotide translocase which is involved in transporting metabolites across the mitochondrial membrane.

One approach to the study of the involvement of phospholipids in particular, in these processes (i.e. translocation and Ca^{2+}-stimulation) is to use lipid-soluble agents which are active in inducing pertubations within the membrane. Compounds in this category include local anesthetics, anti-arrhythmics, tranquilizers and short-chain aliphatic alcohols. A number of these substances have been shown to interact with Ca^{2+} bound to pure phospholipids (18,68), erythrocyte membranes (135) and also mitochondrial membranes (212). On the other hand neutral anesthetics, such as aliphatic alcohols, potentiate the binding of Ca^{2+} to membranes (see refs.224,225).

Mitochondria have been described as a useful model membrane system in which to drug-membrane interactions (29,112,113). They may be isolated in high yield and high purity relatively easily and quickly. They contain a number of enzyme activities which are easy to monitor and finally, these enzyme activities are sometimes analogous in many respects to those occurring in nerve cell membranes, the physiological site of action of most of the membrane-active drugs.

The reverse situation is also true whereby drugs, whose molecular effects have been relatively well characterised, have themselves been used to investigate actual molecular events involved in membrane phenomena.

To this end a number of membrane-active agents have been employed to investigate a variety of mitochondrial functions such as respiration (112,144,182,187,215,218,249), swelling (112,113, 237,238), energy-linked ion movements (7,112,113,167,168,182,187, 215,248,249), cation binding (73,110) and also phospholipid hydrolysis (219,263).

On the other hand relatively few reports have appeared in the literature concerning measurements of the direct binding of membrane-active drugs to mitochondrial membranes (83,106,143, 144,223).

On the basis of the above considerations and particularly in the light of the Ca^{2+}-stimutation of ATP translocation it was decided to undertake a study of the effect of membrane-active agents on the adenine nucleotide translocase. In this way two effects of these substances could be investigated in the one system. These are the effect of membrane pertubations induced by these agents on the translocase activity *per se*, and secondly, the susceptibility of Ca^{2+}-stimulation to alteration of Ca^{2+} binding properties of the mitochondrial membranes.

Local anesthetics, and indeed other membrane-active drugs, stabilise erythrocyte membranes towards osmotic haemolysis at concentrations comparable to those anesthetizing nerve fibres(214). A similar effect is observed for mitochondrial Ca^{2+} accumulation which is stimulated by low 'stabilizing' concentrations of local anesthetics (167). An important property of membrane stabilizing agents is their biphasic mode of action; at high concentrations they become lytic in nature.

It has been suggested that the majority of membrane-active agents interact with mitochondrial membranes by way of combining with negatively charged groups located therein, in addition to intercalating in the phospholipid matrix as a result of their general hydrophobic nature (106). This interaction would lead to altered conformational and physical properties of the membrane, which would be seen in an altered translocase capacity and properties.

This chapter extends the study of the translocase and reports on work carried out with a variety of membrane-active agents and drugs. Particular emphasis is placed on the Ca^{2+}-mediated stimulation of the translocation process. In the first section an examination of the effect of typical local anesthetics on the translocase is made. In the case of butacaine this investigation is more detailed due to the unique inhibitory properties found with this compound. Following sections extend this study to include a variety of membrane-active compounds including alcohols, hypnotics, anti-inflammatories and anti-arrhythmics. Finally the observed effects of these drugs on the translocation of adenine nucleotides is discussed in terms of membrane structure and lipid-protein interactions. Comments on the applicability of results obtained with a mitochondrial membrane system to nerve cell and other membranes are also noted.

Results

Influence of local anesthetics on adenine nucleotide translocation

Four local anesthetics nupercaine, tetracaine, butacaine and procaine, all with an essentially similar basic structure (see Figure 1) were tested for their effects on the translocation of ATP in the absence and presence of Ca^{2+}. An interesting observation from the data presented in Figure 2 was the inhibition of the translocation process by low concentrations of butacaine (K_i approximately 30μM in the presence and absence of Ca^{2+}). Inhibition was also noted using nupercaine and tetracaine but this occurred at higher concentrations and was accompanied by progressive lysis of the mitochondria as indicated by leakage of endogenous adenine nucleotides, morphology of the mitochondrial pellet and substantiated by swelling experiments at 520nM (results not shown). However, concentrations of up to 1mM tetracaine resulted in a stimulation of the translocation of ATP especially in the absence of Ca^{2+} where the stimulation was 50% over that of the control compared to 20% in the presence of Ca^{2+}. The stimulatory effect of Ca^{2+} on ATP translocation was abolished by increasing concentrations of the local anesthetics as indicated by the inserts to Figure 2 where the relative activation for Ca^{2+} (R_{act}) is plotted against the anesthetic concentration. Both tetracaine and butacaine increased this value when present at low concentrations; with the former it increased from 2 to 2·25 and with the latter from 2 to 2·5. Concentrations of the drugs necessary to decrease the difference between this ratio and unity by 50% (K_i) were 200μM, 250μM, 1·7mM and 3·3mM for nupercaine, tetracaine, butacaine and procaine respectively.

Figure 3 shows the results of a similar experiment to that in Figure 2 except that the adenine nucleotide being investigated was ADP. Ca^{2+} ions do not stimulate the translocation of ADP to the same extent as that of ATP; approximately 50% as opposed to 100% under these conditions. Again the responses to the anesthetics were complex, even more so than those observed for ATP. The only similarity between ADP and ATP was with respect to butacaine where again low concentrations produced a large decrease in the rate of translocation with a K_i of approximately 10μM. Increasing concentrations of tetracaine and especially nupercaine produced an

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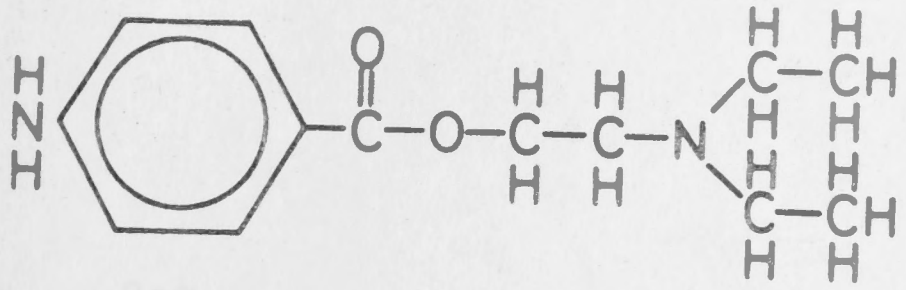

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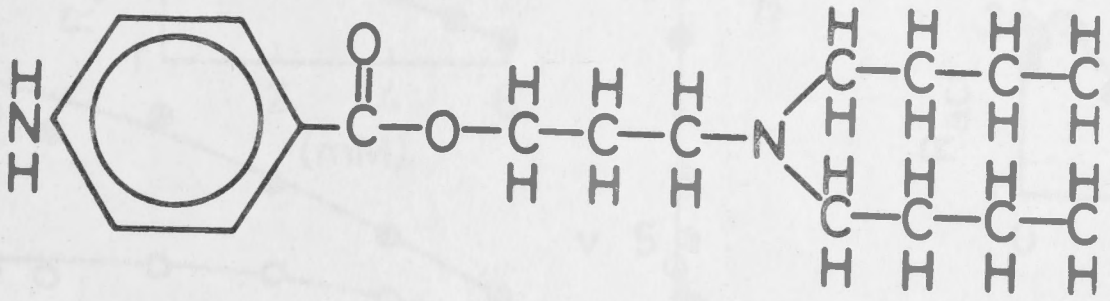

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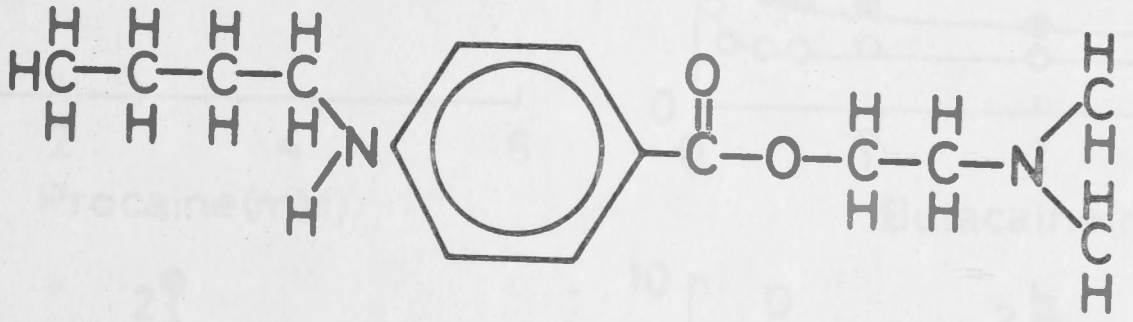

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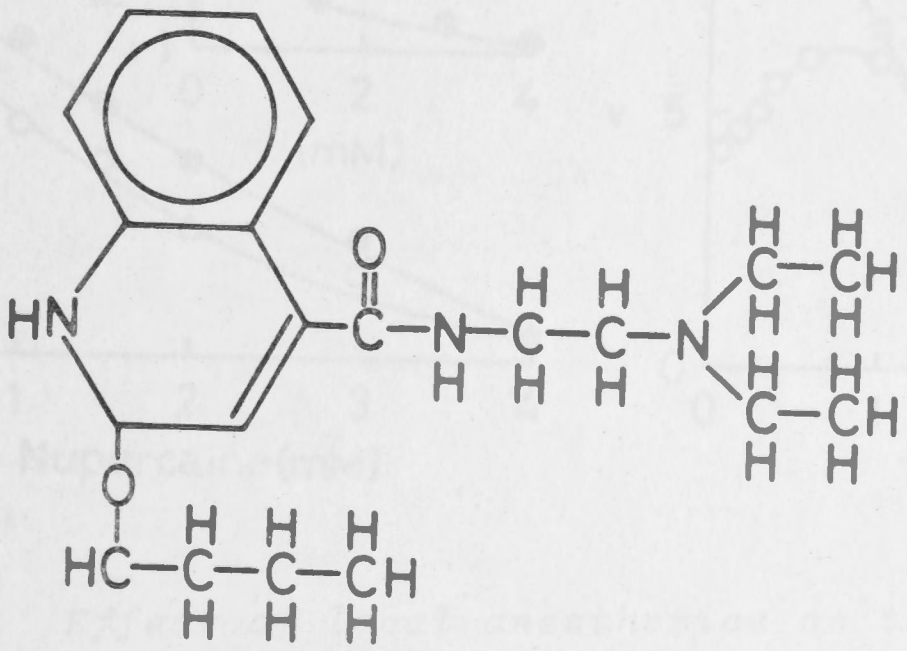


Figure 1. *Structure of local anesthetics.*

A, Procaine; B, Butacaine; C, Tetracaine; D,Nupercaine.

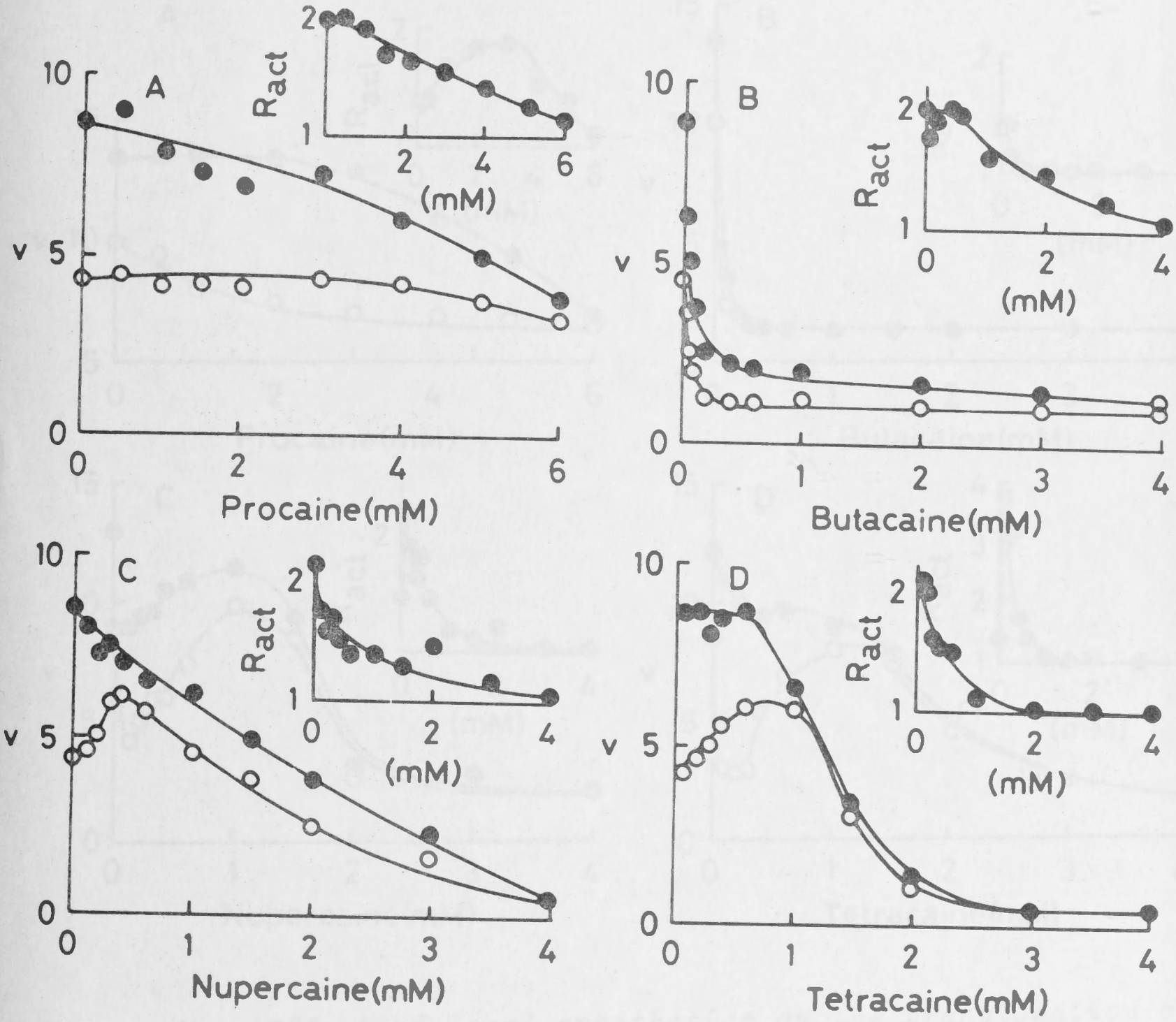


Figure 2. *Effect of local anesthetics on the translocation of ATP.*

Mitochondria were incubated as in method (b) except that a two minute preincubation period with the agent to be tested was employed. The reaction was initiated by the addition of 200μM ATP and 200μM Ca^{2+} as indicated. A, Procaine; B, Butacaine; C, Nupercaine; D, Tetracaine. ○ , no addition; ● , Ca^{2+}. Inserts show the relative activation by Ca^{2+} of ATP translocation (R_{act}) calculated from the data given. v refers to the rate of adenine nucleotide translocation in nmoles/min per mg protein.

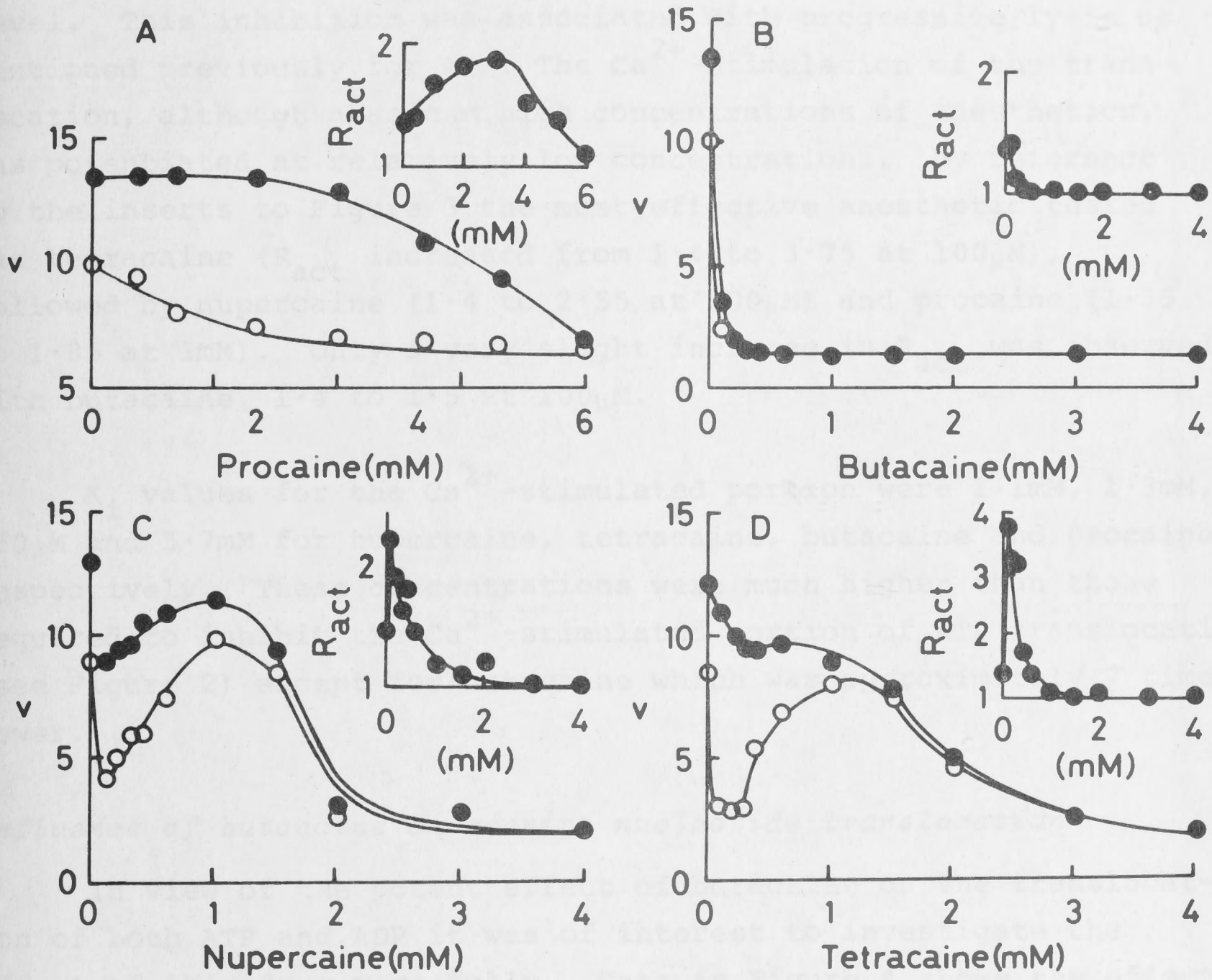


Figure 3. *Effect of local anesthetics on the translocation of ADP.*

Mitochondria were incubated as in the legend to Figure 2 except that the translocation of 200μM ADP was tested. A, Procaine; B, Butacaine; C, Nupercaine; D, Tetracaine. ○ , no addition; ● , 200μM Ca^{2+}. Inserts show the R_{act} induced by Ca^{2+} as a function of anesthetic concentration. v refers to the rate of adenine nucleotide translocation in nmoles/min per mg protein.

oscillatory response. There was an initial inhibition, of up to 65% with tetracaine minus Ca^{2+}, followed by stimulation to the original rate, with the exception of tetracaine plus Ca^{2+} where the effect was less pronounced, and then inhibition to a basal level. This inhibition was associated with progressive lysis as mentioned previously for ATP. The Ca^{2+}-stimulation of the translocation, although absent at high concentrations of anesthetics, was potentiated at relatively low concentrations. By reference to the inserts to Figure 3 the most effective anesthetic tested was tetracaine (R_{act} increased from 1·4 to 3·75 at 100μM), followed by nupercaine (1·4 to 2·55 at 100μM) and procaine (1·35 to 1·85 at 3mM). Only a very slight increase in R_{act} was observed with butacaine, 1·4 to 1·5 at 100μM.

K_i values for the Ca^{2+}-stimulated portion were 1·1mM, 1·3mM, 250μM and 5·7mM for nupercaine, tetracaine, butacaine and procaine respectively. These concentrations were much higher than those required to inhibit the Ca^{2+}-stimulated portion of ATP translocation (see Figure 2) except for butacaine which was approximately 7 times lower.

Influence of butacaine on adenine nucleotide translocation

In view of the potent effect of butacaine on the translocation of both ATP and ADP it was of interest to investigate the effect of this drug more fully. Data in Figure 4 shows the effect of butacaine concentration on the translocation rate of different concentrations of ATP (10 to 400μM). Stimulation of ATP translocation is observed when the concentrations of both added ATP and of butacaine were low; up to about 50μM of each compound. The degree of this stimulation decreased with increasing ATP concentration from 78% at 10μM ATP such that it is abolished at 100μM ATP (see Table I); the respective concentrations for ½ maximal stimulation (K_a) also decreased, from 15μM at 10μM ATP to 6μM at 50μM ATP. Inhibition observed with higher butacaine concentrations is not complete and reaches a basal level of approximately 0.6 to 0.8 nmoles/mg protein/min and is virtually independent of the ATP concentration employed. Consequently the maximal % inhibition increases from 30% at 10μM ATP to 85% at 400μM. Moreover, as shown in Table I, the butacaine concentration needed to achieve 50% maximal inhibition (K_i) decreases from 280μM to 28μM at 10 and 400μM ATP respectively.

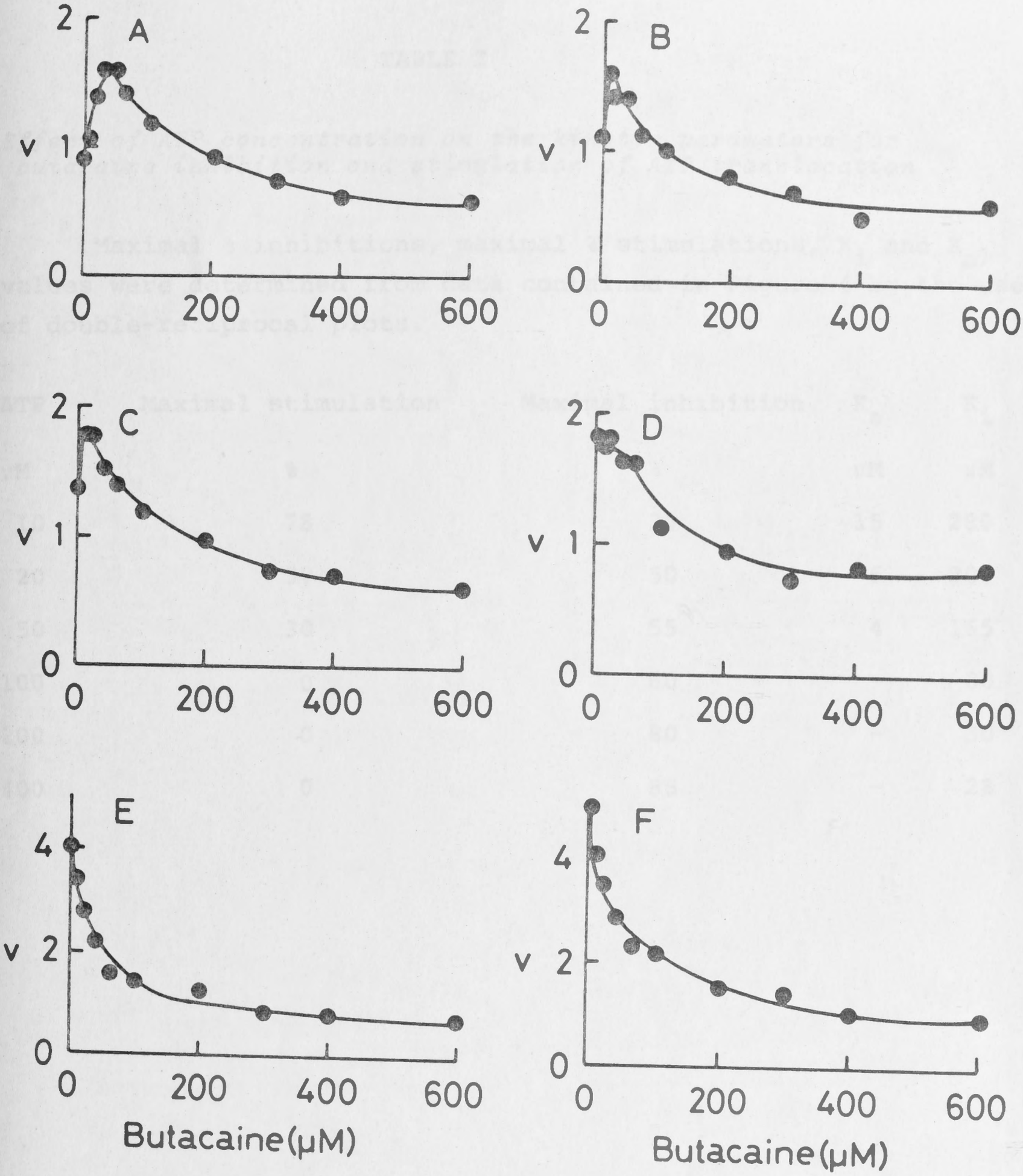


Figure 4. *Effect of butacaine concentration on the translocation of ATP.*

Mitochondria were incubated as described in the legend to Figure 2 with increasing amounts of butacaine and ATP as indicated. A, 10μM ATP; B, 20μM; C, 50μM; D, 100μM; E, 200μM; F, 400μM. v refers to the rate of ATP translocation in nmoles/min per mg protein.

TABLE I

Effect of ATP concentration on the kinetic parameters for butacaine inhibition and stimulation of ATP translocation

Maximal % inhibitions, maximal % stimulations, K_i and K_a values were determined from data contained in Figure 4 by the use of double-reciprocal plots.

ATP	Maximal stimulation	Maximal inhibition	K_a	K_i
μM	%	%	μM	μM
10	78	30	15	280
20	50	50	6	200
50	30	55	4	165
100	0	60	-	80
200	0	80	-	30
400	0	85	-	28

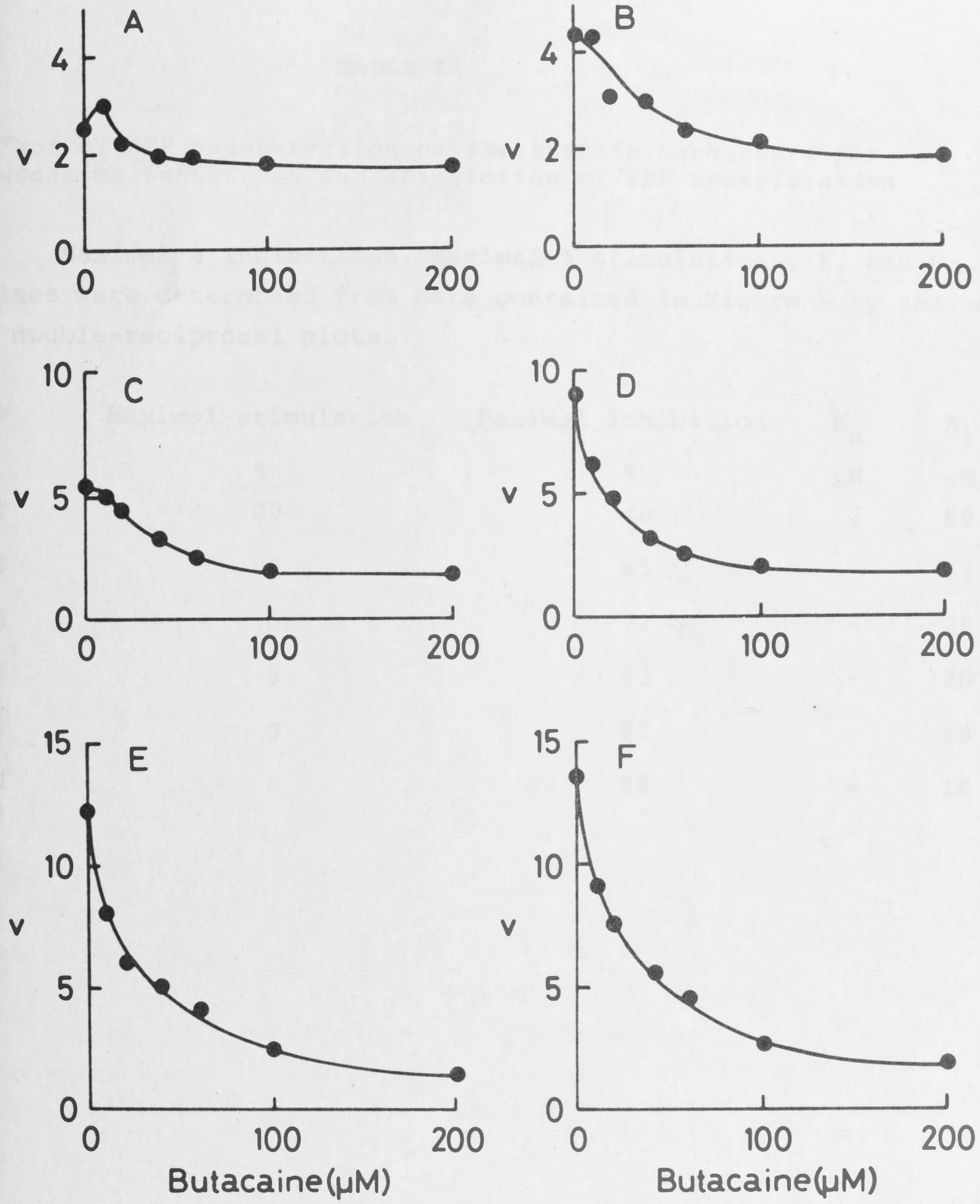


Figure 5. *Effect of butacaine concentration on the translocation of ADP.*

Mitochondria were incubated as described in the legend to Figure 3 with increasing amounts of butacaine and ADP as indicated. A, 10μM ADP, B, 20μM; C, 50μM; D, 100μM; E, 200μM; F, 400μM. v refers to the rate of ADP translocation in nmoles/min per mg protein.

TABLE II

Effect of ADP concentration on the kinetic parameters for butacaine inhibition and stimulation of ADP translocation

Maximal % inhibitions, maximal % stimulations, K_i and K_a values were determined from data contained in Figure 5 by the use of double-reciprocal plots.

ADP	Maximal stimulation	Maximal inhibition	K_a	K_i
µM	%	%	µM	µM
10	20	40	5	60
20	0	65	-	32
50	0	72	-	28
100	0	83	-	20
200	0	85	-	18
400	0	86	-	18

Data presented in Figure 5 shows the effect of butacaine concentration on the translocation of increasing concentrations of ADP. Again stimulation was observed at low ATP levels but this stimulation was not as great as that for ATP (see Figure 4) and was not seen above 10$_\mu$M ADP. Maximal % inhibition increased from 40% at 10$_\mu$M ADP to 86% at 400$_\mu$M. Basal translocation rates varied from 1·5 to 1·8 mol per mg protein per min over the range of ADP concentrations tested. As in the case of ATP the K_i for butacaine decreased with increasing ADP from 60$_\mu$M to 18$_\mu$M over the range of ADP concentrations employed (see Table II).

Effect of butacaine on the affinity of the translocase for adenine nucleotide

The effect of a constant concentration of butacaine (50$_\mu$M) on the translocation of ATP measured as a function of ATP concentration is shown in Figure 6a. As predicted from the data presented in Figure 3 at low ATP concentrations butacaine is stimulatory and at higher concentrations it is inhibitory. A similar type of experiment is shown in Figure 6b for ADP. In this case butacaine is inhibitory at all ADP concentrations tested. A more direct comparison between the effect of butacaine on ATP and ADP translocation is seen when these data are replotted in a double-reciprocal form so as to compare the K_m and V_{max} values (see Figures 7a and b). The Lineweaver-Burk plot shows non-linearity, indicating two processes in the translocation reaction, from which two apparent K_m values may be obtained. Data in Table III summarises the kinetic constants obtained from these two plots as well as those obtained using several lower concentrations of butacaine. As the concentration of butacaine is increased from zero to 50$_\mu$M both the high and low K_m's for ATP decrease until the former becomes non-existent whilst the latter falls from 15$_\mu$M to 5$_\mu$M, i.e. the affinity for ATP is increased some three-fold.

With ADP the high K_m increases from 50$_\mu$M to 100$_\mu$M and the low K_m decreases by 25% from 8$_\mu$M to 6$_\mu$M as the butacaine concentration is increased to 50$_\mu$M. Thus in the presence of 50$_\mu$M butacaine the affinity of the translocase for both ATP and ADP is approximately equivalent. ATP translocation seems to be more sensitive to inhibition by butacaine than ADP as evidenced by greater % inhibition of the former. V_{max} values decrease by 78% from 7·7 to 2·25 nmol per mg protein per min and by 52% from 14·7 to 7·0 nmol per mg protein per min for ATP and ADP respectively.

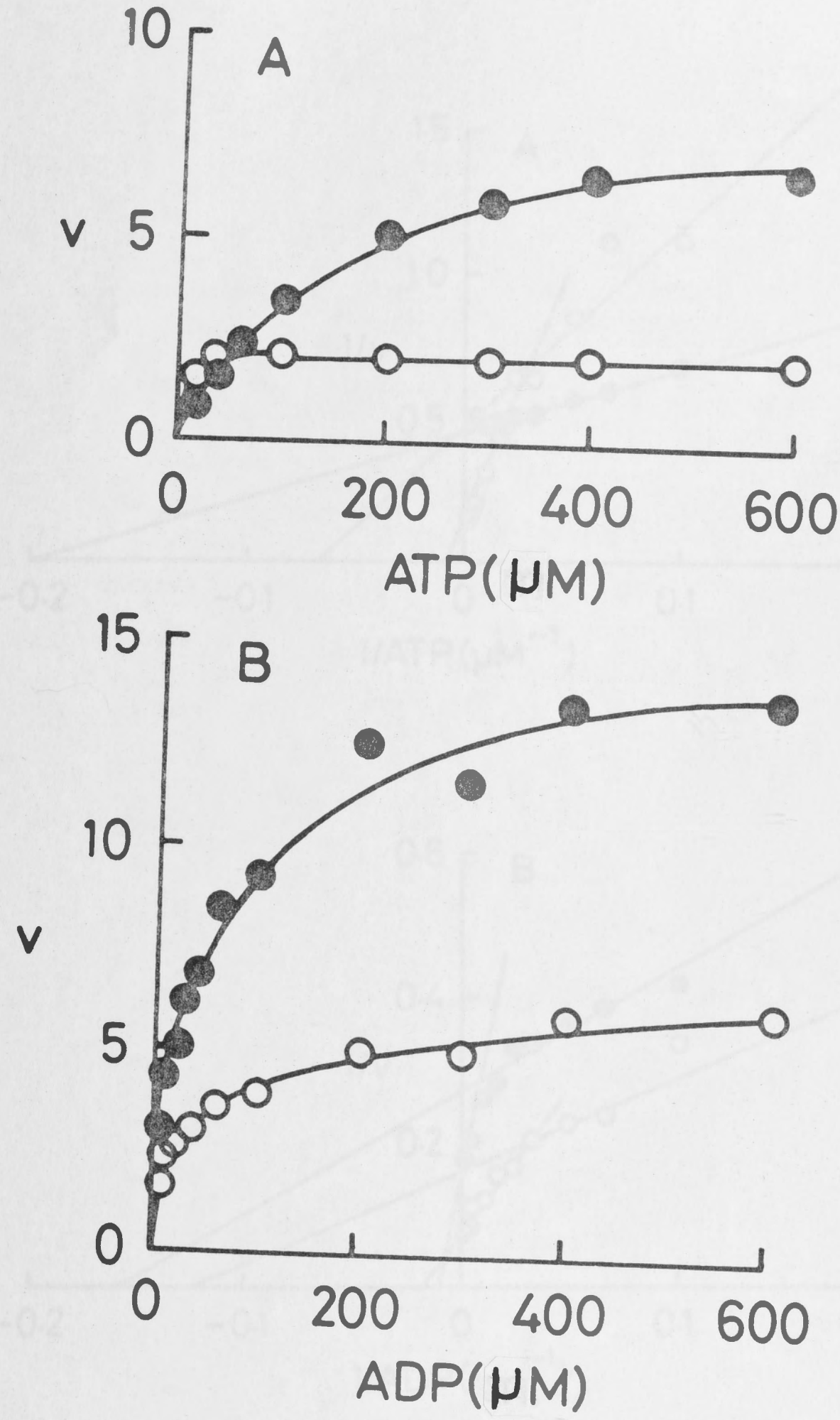
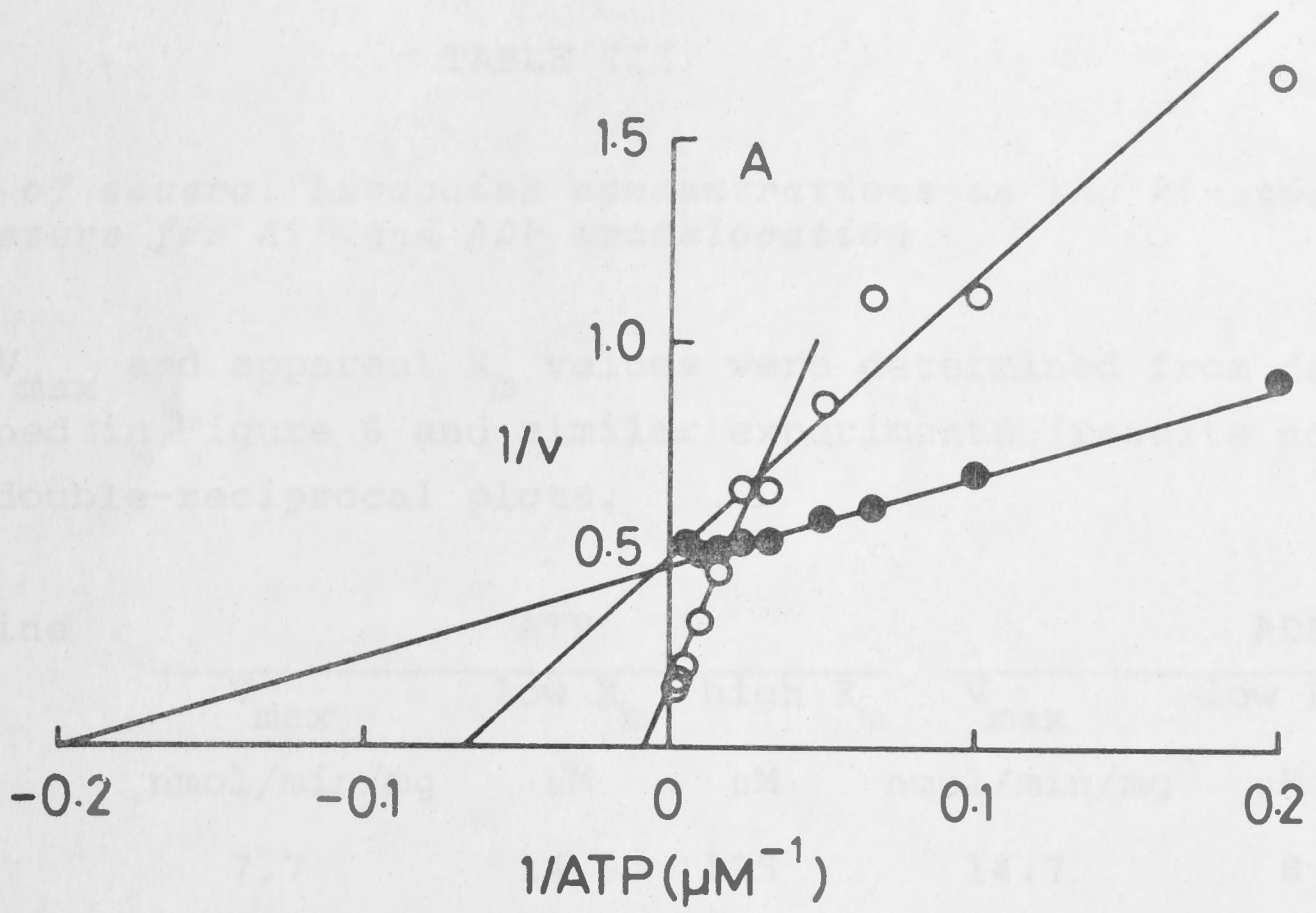


Figure 6. *Effect of butacaine on the translocation of adenine nucleotides.*

Mitochondria were incubated as described in the legend to Figure 2 with increasing amounts of ATP or ADP and 50μM butacaine as indicated. A, ATP; B, ADP. Closed symbols, no addition; open symbols, butacaine. v refers to the rate of adenine nucleotide translocation in nmoles/min per mg protein.



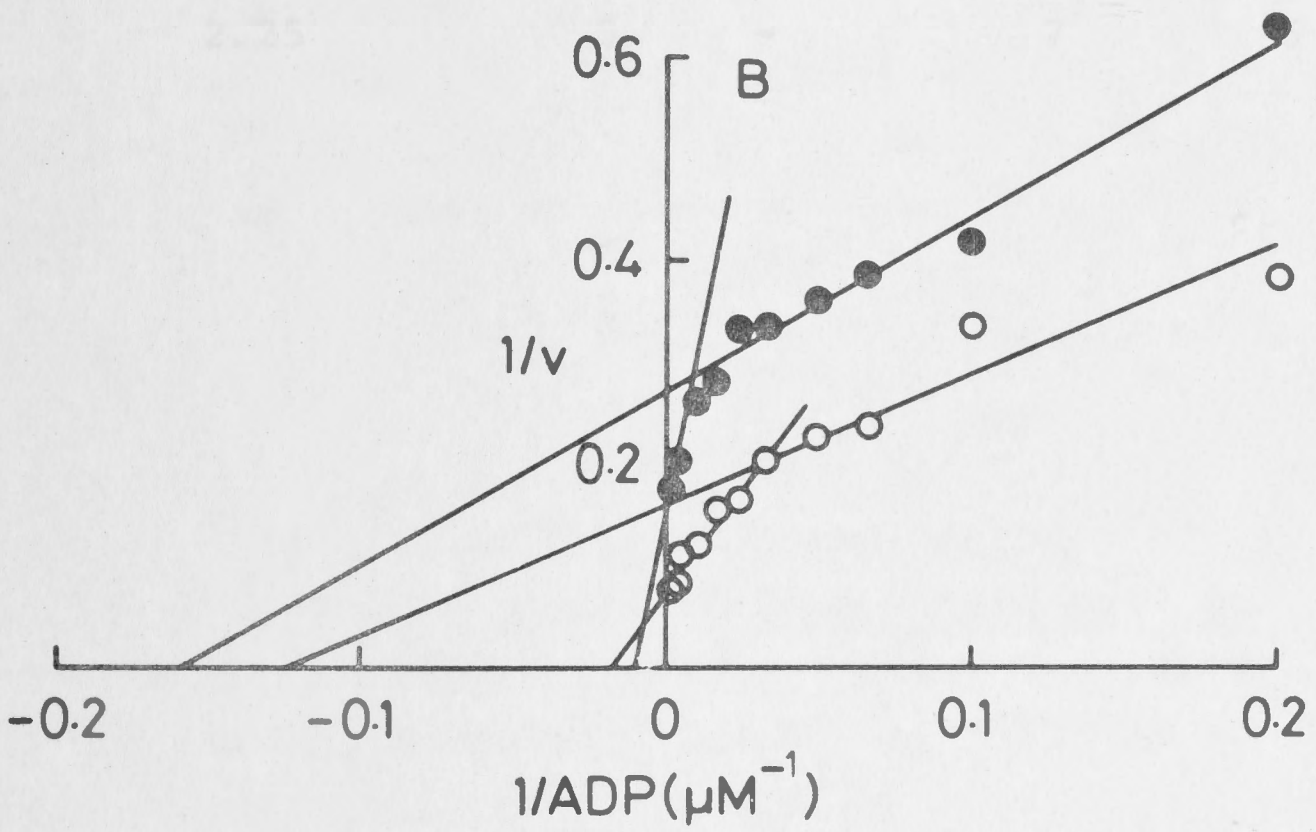


Figure 7. *Double reciprocal plot of the data contained in Figure 6.*

A, ATP; B, ADP. Open symbols, no addition; closed symbols, 50μM butacaine.

TABLE III

Effect of several butacaine concentrations on the kinetic parameters for ATP and ADP translocation

V_{max} and apparent K_m values were determined from data contained in Figure 6 and similar experiments (results not shown) using double-reciprocal plots.

Butacaine	ATP			ADP		
added	V_{max}	low K_m	high K_m	V_{max}	low K_m	high K_m
µM	nmol/min/mg	µM	µM	nmol/min/mg	µM	µM
0	7.7	14.5	125	14.7	8	50
10	5.6	14	83	11	8	70
20	3.7	13	45	9	7	85
50	2.25	5	-	7	6	100

Influence of local anesthetics on the translocation of a low ATP concentration

Data in Figure 8 show the effect of nupercaine, tetracaine and procaine on the translocation of 10μM ATP. Previously it had been shown that the translocation rate is stimulated using low butacaine concentrations under these conditions whilst inhibition occurred when higher concentrations were present (see Figure 4). This stimulation/inhibition phenomenon was mimicked by both nupercaine and tetracaine. There is one difference, however, in that the inhibition did not result in a return to a rate equal to or less than the rate in the basal state, i.e. in the absence of anesthetics. Procaine also stimulated the translocation with a plateau being observed at 5mM, the highest concentration tested. Presumably at concentrations higher than this inhibition would be present. Kinetic data calculated from these plots (Table IV) showed that maximal stimulation was approximately the same for butacaine, nupercaine and tetracaine (77% to 85%) but in the case of procaine this value was increased to 200%. Apparent K_m's for this stimulation were 10μM, 12μM, 15μM and 90μM for nupercaine, tetracaine, butacaine and procaine respectively.

Influence of pH on butacaine inhibition of adenine nucleotide translocation

In order to further characterise the effects of butacaine the inhibition was studied using incubation media of varying pH. Data in Figure 9 shows the effect of pH varied over the range 5·4 to 8·4 on the kinetic parameters for this inhibition. With both ATP and ADP there was an optimum inhibition at pH 7·4 as indicated by the calculated maximal % inhibitions. These values varied from 55% to 90% and from 57% to 79% for ATP and ADP respectively. With both ADP and ATP no significant change occurred in the K_i; these values varied from 20μM to 23μM for ADP and from 40μM to 46μM for ATP. Attempts were made to correlate these changes as a function of pH with changes in the proportion of the ionic species of butacaine present in the incubation medium. Results of a pH titration curve for butacaine (Figure 10) indicated that this proportion did not correlate with the observed pH effect on maximal inhibition by butacaine of the translocation process. Calculated pK values from this plot are approximately 2·5 and 8·6.

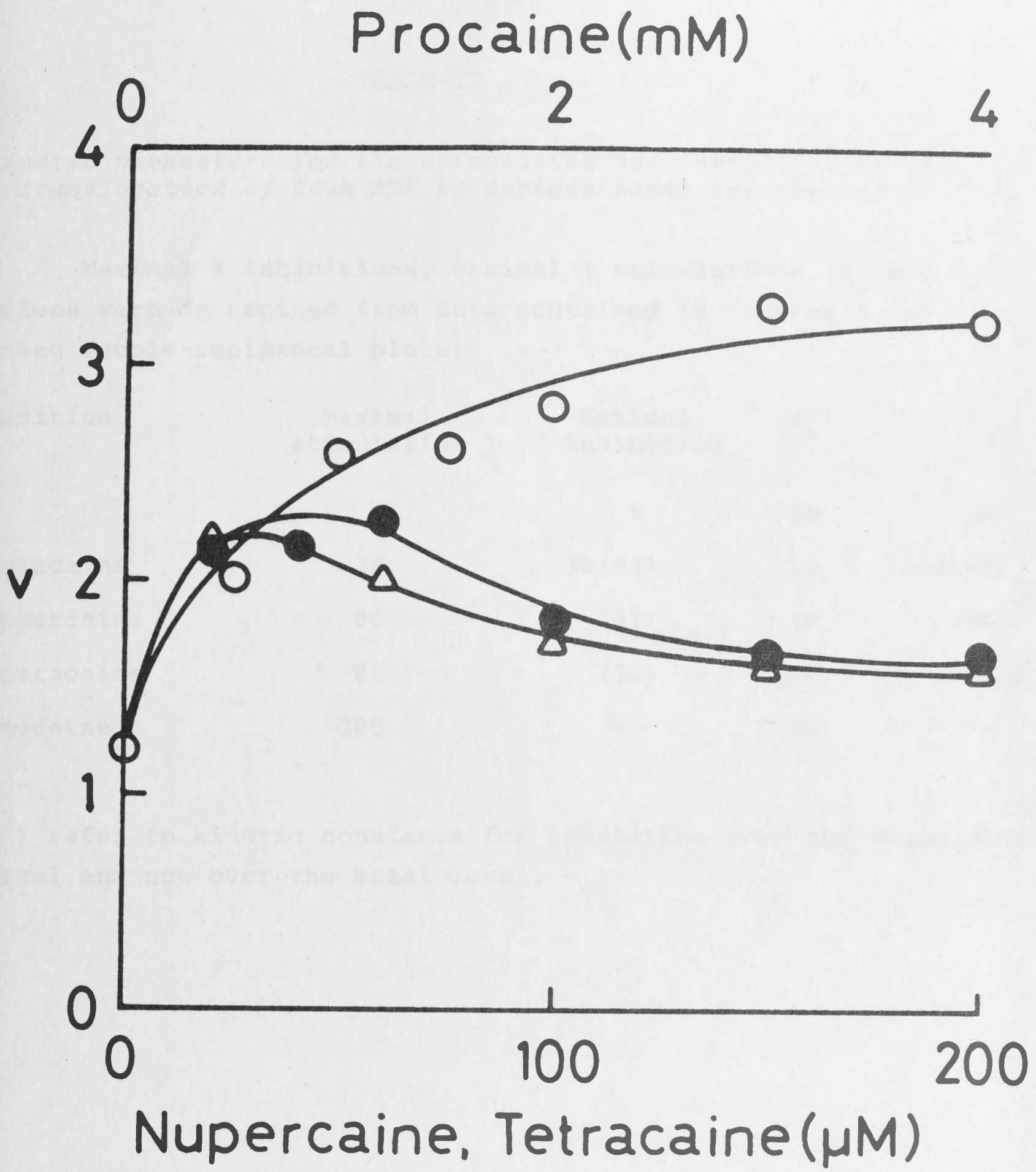


Figure 8. *Effect of local anesthetics on the translocation of 10μM ATP.*

Mitochondria were incubated as described in the legend to Figure 2 with 10μM ATP and increasing amounts of local anesthetic as indicated. ○ , procaine; ● , tetracaine; △ , nupercaine. v refers to the rate of adenine nucleotide translocation in nmoles/min per mg protein.

TABLE IV

Kinetic parameters for the stimulation and inhibition of the translocation of 10μM ATP by various local anesthetics

Maximal % inhibitions, maximal % stimulations, K_i and K_a values were determined from data contained in Figures 4 and 7 using double-reciprocal plots.

Addition	Maximal stimulation	Maximal inhibition	K_a	K_i
	%	%	μM	μM
Butacaine	78	30(63)	15	280(120)
Nupercaine	80	(27)	10	(80)
Tetracaine	85	(32)	12	(90)
Procaine	200	-	90	-

( ) refer to kinetic constants for inhibition over the stimulated level and not over the basal level.

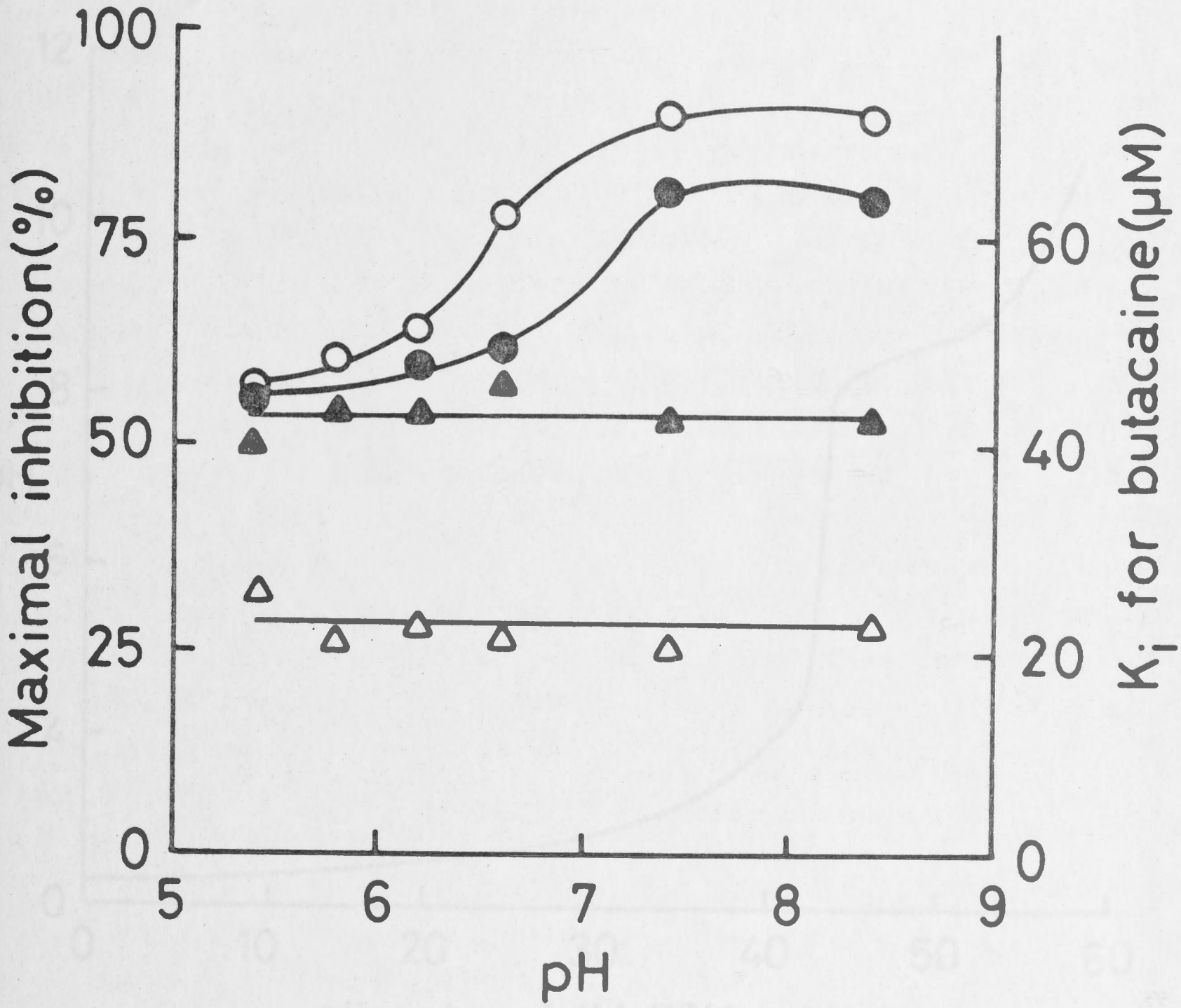


Figure 9. *Effect of pH on the apparent K_i and % maximal inhibition of adenine nucleotide translocation by butacaine.*

Mitochondria were incubated as described in the legend to Figure 2 at the pH values indicated with 200μM ADP (open symbols) or ATP (closed symbols) and various concentrations of butacaine. The kinetic constants were determined using double-reciprocal plots. Triangles, K_i values; circles, % maximal inhibition.

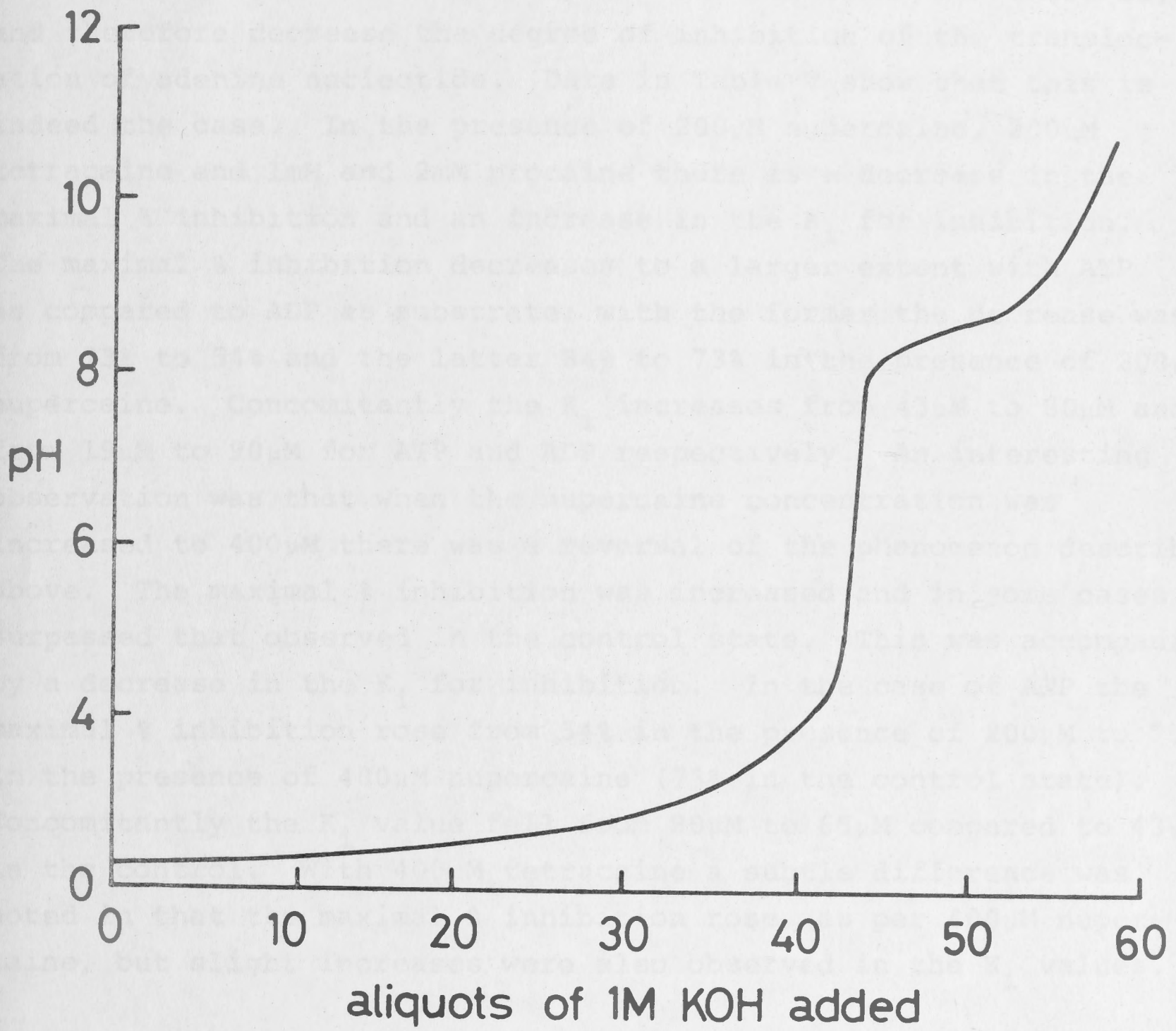


Figure 10. *pH titration curve for butacaine.*

A 2mM solution of butacaine was brought to pH 2 using 5M HCl and then back-titrated using aliquots of 2M KOH. The pH reading after each of the KOH additions was then plotted against the number of additions. pK values were estimated from the midpoints of the plateaux.

Influence of local anesthetics on butacaine inhibition of adenine nucleotide translocation

It is probable that there are common binding site(s) on the mitochondrial membrane for all local anesthetics tested (see ref.106). Thus it would be expected that nupercaine, tetracaine and procaine at concentrations which do not appreciably alter the rate of translocation would compete with butacaine for these sites and therefore decrease the degree of inhibition of the translocation of adenine nucleotide. Data in Table V show that this is indeed the case. In the presence of 200μM nupercaine, 200μM tetracaine and 1mM and 2mM procaine there is a decrease in the maximal % inhibition and an increase in the K_i for inhibition. The maximal % inhibition decreases to a larger extent with ATP as compared to ADP as substrate; with the former the decrease was from 73% to 54% and the latter 84% to 73% in the presence of 200μM nupercaine. Concomitantly the K_i increases from 43μM to 80μM and from 19μM to 90μM for ATP and ADP respectively. An interesting observation was that when the nupercaine concentration was increased to 400μM there was a reversal of the phenomenon described above. The maximal % inhibition was increased and in some cases surpassed that observed in the control state. This was accompanied by a decrease in the K_i for inhibition. In the case of ATP the maximal % inhibition rose from 54% in the presence of 200μM to 75% in the presence of 400μM nupercaine (73% in the control state). Concomitantly the K_i value fell from 80μM to 65μM compared to 43μM in the control. With 400μM tetracaine a subtle difference was noted in that the maximal % inhibition rose, as per 400μM nupercaine, but slight increases were also observed in the K_i values.

In view of the imcomplete reversal of the butacaine inhibition shown above by the inclusion of 200μM nupercaine the effect of preincubating the mitochondria for 2 minutes with this local anesthetic before the addition of the butacaine was investigated. As shown by the data nupercaine was more efficient under these conditions in negating the inhibition by butacaine; the maximal % inhibition decreased from 73% to 63% whilst the K_i rose from 90μM to 100μM indicating a slightly lower affinity for butacaine.

Influence of K^+ and La^{3+} on butacaine inhibition

Both local anesthetics and K^+ ions are thought to interact with mitochondrial membranes and compete with Ca^{2+} for Ca^{2+}

TABLE V

Effect of nupercaine, tetracaine and procaine on the inhibition by butacaine of adenine nucleotide translocation

Mitochondria were incubated as described in the legend to Figure 2 with increasing concentrations of butcaine, 200μM ATP or ADP, 200μM Ca^{2+} when present and other local anesthetics as indicated. When a preincubation period was used 200μM Nupercaine was added to the incubation medium containing the mitochondria 2 minutes before the addition of the appropriate butacaine concentration. Maximal % inhibitions and K_i values were determined using double reciprocal plots.

Adenine nucleotide tested		Control	Nupercaine		Tetracaine		Procaine	
			200μM	400μM	200μM	400μM	1mM	2mM
(i) ATP	(Max % inhb.	73	54	75	65	68	69	55
	(K_i (μM)	43	80	65	90	160	46	55
ATP+Ca^{2+}	(Max % inhb.	77	53	59	41	65	73	46
	(K_i (μM)	36	130	95	60	240	45	120
ADP	(Max % inhb.	84	73	91	63	68	79	68
	(K_i (μM)	19	90	58	38	42	38	42
ADP+Ca^{2+}	(Max % inhb.	81	72	90	75	78	78	75
	(K_i (μM)	25	72	50	45	50	50	65
(ii) ADP	(Max % inhb.	89	63	-	-	-	-	-
	(K_i (μM)	20	100	-	-	-	-	-

TABLE VI

Effect of K^+ and La^{3+} on the inhibition of adenine nucleotide translocation by butcaine

Mitochondria were incubated as described in the legend to Figure 2 with increasing amounts of butacaine and 200μM ATP or ADP. Ca^{2+}, K^+ and La^{3+} when present were at concentrations of 200μM, 20mM and 50μM, respectively. Maximal % inhibitions and K_i values were determined using double-reciprocal plots.

Adenine nucleotide tested		Maximal inhibition %	K_i μM
ATP	(-	75	42
	(+ K^+	67	90
	(+ La^{3+}	79	50
ATP+Ca^{2+}	(-	77	36
	(+ K^+	75	200
ADP	(-	88	19
	(+ K^+	74	90
	(+ La^{3+}	89	22
ADP+Ca^{2+}	(-	81	27
	(+ K^+	78	190

binding sites (7,112,212,215). Thus it would be expected that K^+ and Ca^{2+} would also interfere with each other in their interactions with these membranes. Data in Table VI shows the effects of 20mM KCl on the kinetic parameters obtained for the inhibition of adenine nucleotide translocation by butacaine. In all cases only a slight decrease in the maximal stimulation was obtained over that in the control state. The most striking effect however was the large increase in the K_i values obtained in the presence of K^+. This effect was even more accentuated when Ca^{2+} was also present in the incubation medium. Thus for ATP, the K_i was increased twofold, from 42μM to 90μM, in the absence of Ca^{2+} and fivefold, from 36μM to 200μM, in the presence of 200μM Ca^{2+}. Similar results were obtained with ADP although the effect was not as pronounced.

Another compound which interacts with both membrane-bound (149,214) and isolated phospholipids (178,212) is La^{3+}, a rare earth cation. As for K^+ it would be expected that this cation would interfere with the interaction of butacaine with the mitochondrial membrane and thus the inhibition by this local anesthetic of adenine nucleotide translocation. With this in mind the effect of the addition of 50μM La^{3+} on the translocation of ATP and ADP as a function of butacaine concentration was tested. Data in Table VI indicates that the presence of the La^{3+} did not significantly influence the maximal % inhibition observed (approximately 95% in the case of ADP) nor result in any great change in the affinity for the inhibitor butacaine; the K_i rose from 19μM to 22μM in the presence of La^{3+}. Similar results were also obtained for ATP.

Influence of local anesthetics on butacaine stimulation at a low ATP concentration

The effect of nupercaine, tetracaine and procaine on the stimulation by low concentrations of butacaine on the translocation of 10μM ATP was tested and the results presented in Table VII. In the presence of 200μM and 400μM of both nupercaine and tetracaine the stimulatory effect of butacaine and the consequent inhibition at higher anesthetic concentrations was completely abolished, i.e. increasing butacaine concentration had no effect on the translocation rate. The addition of procaine, however, resulted in

TABLE VII

Effect of nupercaine, tetracaine and procaine on the inhibition and stimulation of the translocation of 10μM ATP by butacaine

Mitochondria were incubated as described in the legend to Figure 2 with increasing amounts of butacaine, 10μM ATP and other local anesthetics as indicated. Maximal % inhibitions, maximal % stimulations, K_i and K_a values were determined using double-reciprocal plots.

Addition	Maximal stimulation	Maximal inhibition	K_a	K_i
-	%	%	μM	μM
-	40	30	8	200
200μM nupercaine	0	0	-	-
200μM tetracaine	0	0	-	-
1mM procaine	0	25	-	200
2mM procaine	0	40	-	12

inhibition of the translocation of ATP such that in the presence of 2mM procaine there was a 40% inhibition with a K_i of 12μM for butacaine. No stimulatory effects were observed on the addition of the low butacaine concentrations.

Influence of pH on butacaine stimulation at a low ATP concentration

As a comparison to the effect of pH on the inhibitory properties of butacaine the effect of pH on the stimulation of the translocation of 10μM ATP by butacaine was also investigated. Data presented in Table VIII indicates that the degree of stimulation decreased slightly under acid conditions; from 39% at pH 7.4 to 32% at pH 5.4. However the K_m for butacaine remained constant at 10μM at all pH values tested. Under these same conditions the maximal % inhibition observable at higher butacaine concentrations (see Figure 4), decreased from 50% to 20% over the pH range 5·8 to 8·4. Concomitantly the K_i increased from 85μM to 250μM.

Effect of aliphatic alcohols

With increasing alcohol concentration the translocation of ATP, both in the absence and presence of Ca^{2+}, was decreased and in most cases eventually became non-existent. Some alcohols, e.g. tert-butanol did not show complete inhibition. However, by analogy with other compounds this would undoubtedly occur when higher concentrations still were added. In the presence of 200μM Ca^{2+} a gradual inhibition of the translocation process was observed with increasing alcohol concentrations. When Ca^{2+} was absent, however, the situation was complicated to some degree by the fact that initially there was no response to the alcohol followed by a maximum in the rate of translocation and thereafter inhibition. Even higher concentration of alcohol resulted in lysis of the mitochondria as indicated by the loss of endogenous adenine nucleotide and decreased O.D. at 520nM (results not shown).

The concentrations of alcohol required to decrease ATP translocation by 50% in the presence of Ca^{2+} ($N_{0.5}$) and to give the maximum rate of translocation in the absence of Ca^{2+} (N_{max}) were plotted against the number of carbon atoms of the n-alcohols (Figure 12a). With increasing number of carbon atoms both values decreased. When this data was plotted in a logarithmic

TABLE VIII

Effect of pH on the inhibition and stimulation of the translocation of 10μM ATP by butacaine

Mitochondria were incubated as described in the legend to Figure 2 with increasing amounts of butacaine, 10 M ATP and the pH varied as indicated. Maximal % inhibitions, maximal % stimulations, K_i and K_a values were determined using double-reciprocal plots.

pH	Maximal stimulation %	Maximal inhibition %	K_a μM	K_i μM
5·5	32	50	10	85
7.4	39	25	10	130
8.4	35	20	10	130

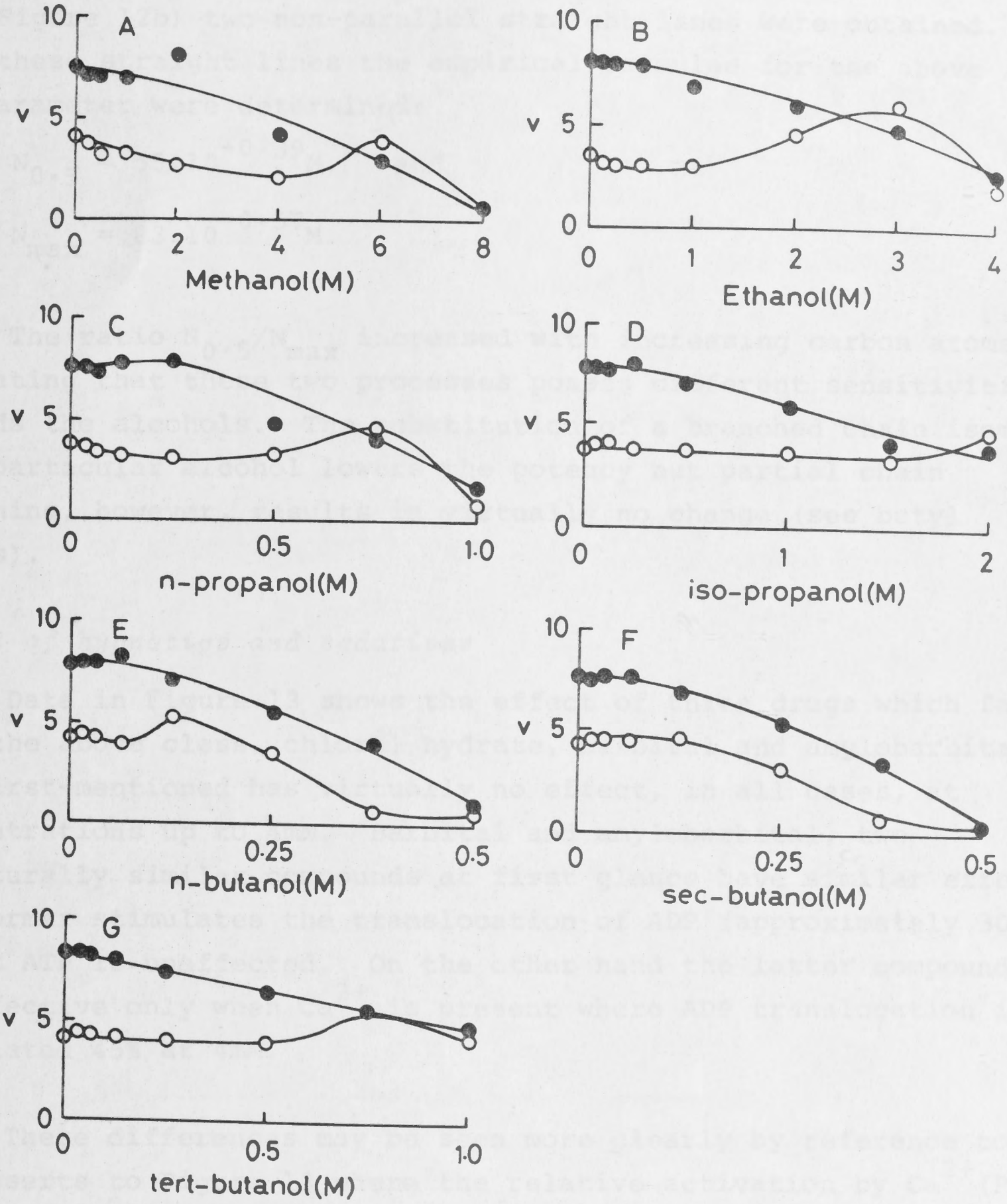


Figure 11. *Effect of alcohols on the translocation of ATP.*

Mitochondria were incubated as described in the legend to Figure 2 with 200μM ATP, various concentrations of alcohols and 200μM Ca^{2+} as indicated. A, Methanol; B, Ethanol; C, n-Propanol; D, iso-Propanol; E, n-Butanol; F, sec-Butanol; G, tert-Butanol. Open symbols, no addition; closed symbols, Ca^{2+}. v refers to the rate of adenine nucleotide translocation in nmoles/min per mg protein.

form (Figure 12b) two non-parallel straight lines were obtained. From these straight lines the empirical formulae for the above two parameter were determined:

$$N_{0\cdot5} = 55.10^{-0\cdot39}M, \quad \text{and}$$

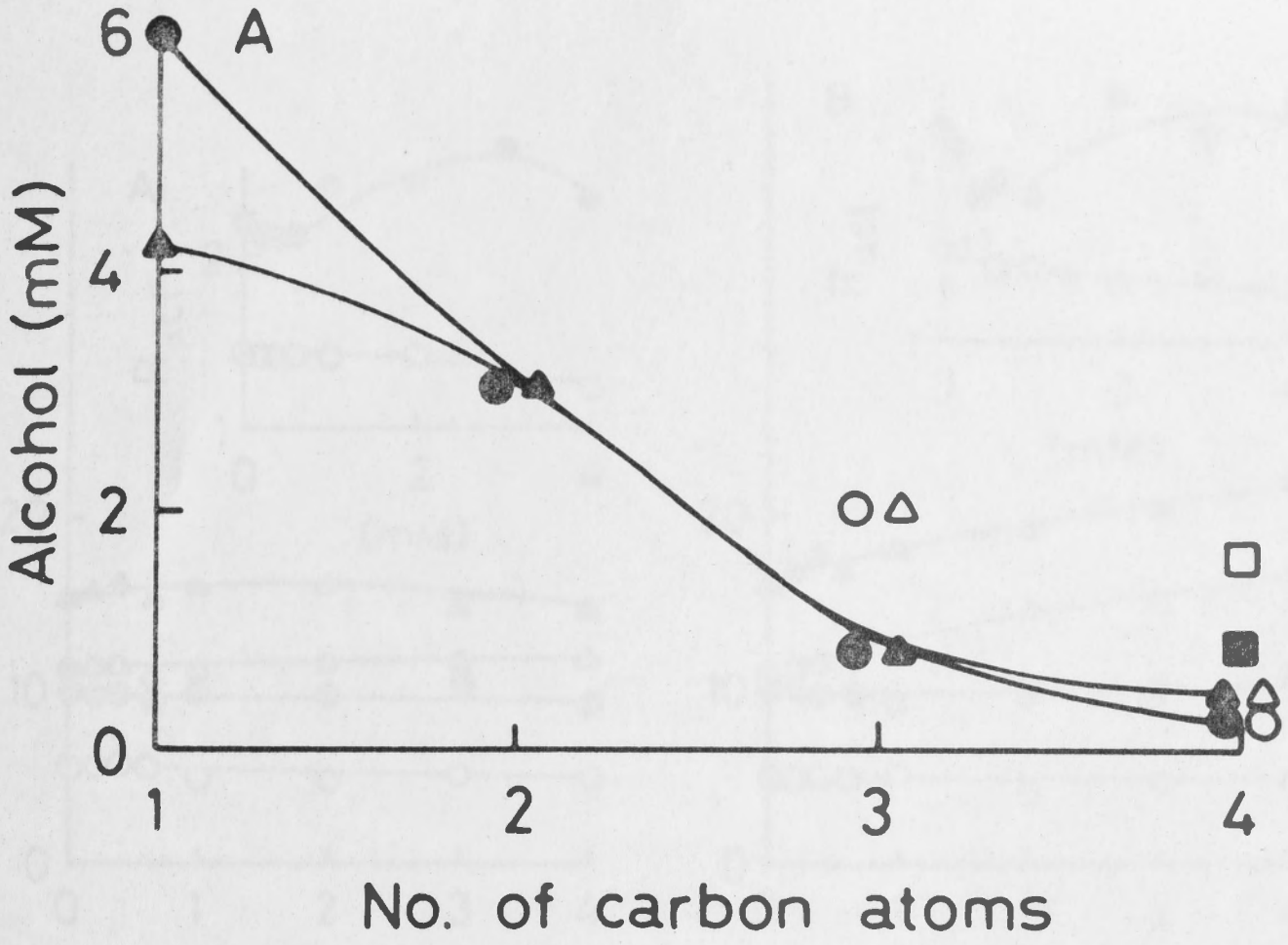
$$N_{max} = 83.10^{-0\cdot57}M.$$

The ratio $N_{0\cdot5}/N_{max}$ increased with increasing carbon atoms indicating that these two processes posses different sensitivities towards the alcohols. The substitution of a branched chain isomer of a particular alcohol lowers the potency but partial chain branching, however, results in virtually no change (see butyl series).

Effect of hypnotics and sedatives

Data in Figure 13 shows the effect of three drugs which fall into the above class, chloral hydrate, barbital and amylobarbital. The first-mentioned has virtually no effect, in all cases, at concentrations up to 4mM. Barbital and amylobarbital, two structurally similar compounds at first glance have similar effects. The former stimulates the translocation of ADP (approximately 30%) whilst ATP is unaffected. On the other hand the latter compound is effective only when Ca^{2+} is present where ADP translocation is stimulated 45% at 4mM.

These differences may be seen more clearly by reference to the inserts to Figure 13 where the relative activation by Ca^{2+} (R_{act}) is plotted against drug concentration. Several points are evident from this data. Firstly, amylobarbital potentiates the Ca^{2+}-stimulation of ADP translocation (R_{act} increases from 1·5 to 2·0) whilst barbital virtually has no effect. Chloral hydrate slightly decreases this stimulation by Ca^{2+} with R_{act} falling from 1·4 to 1·2. Secondly, the addition of low concentrations of amylobarbital results in a slight increase of the Ca^{2+}-stimulation of ATP translocation whilst barbital produces the opposite effect. Similar stimulations are observable at chloral hydrate concentrations above approximately 1mM.



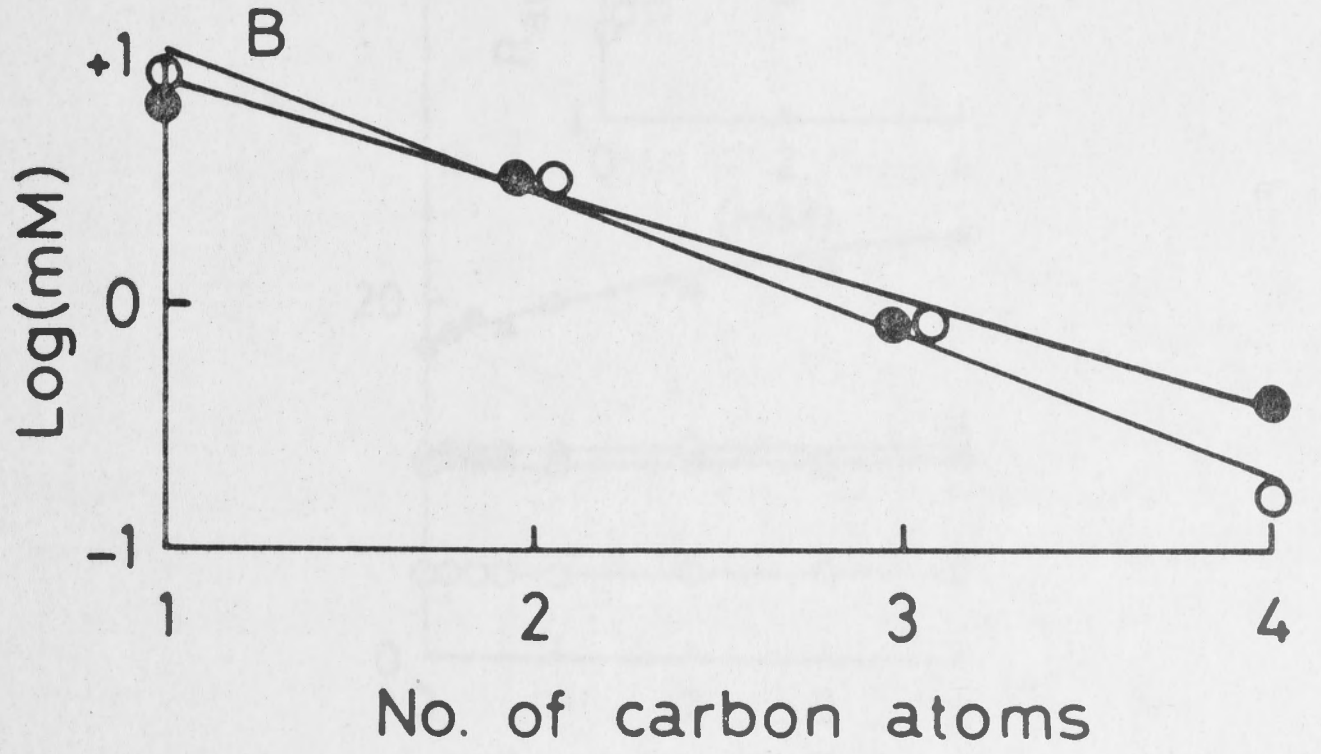


Figure 12. *Alcohol concentrations required to affect ATP translocation.*

A, linear plot; B, logarithmic plot. Triangles and open square, concentration required for 50% inhibition of Ca^{2+}-stimulated translocation; circles and closed squares, concentration required to maximally stimulate ATP translocation in the absence of Ca^{2+}. Closed circles and triangles, n-alcohols; open circles and triangles, iso- or sec-alcohols. Squares, tert-butanol.

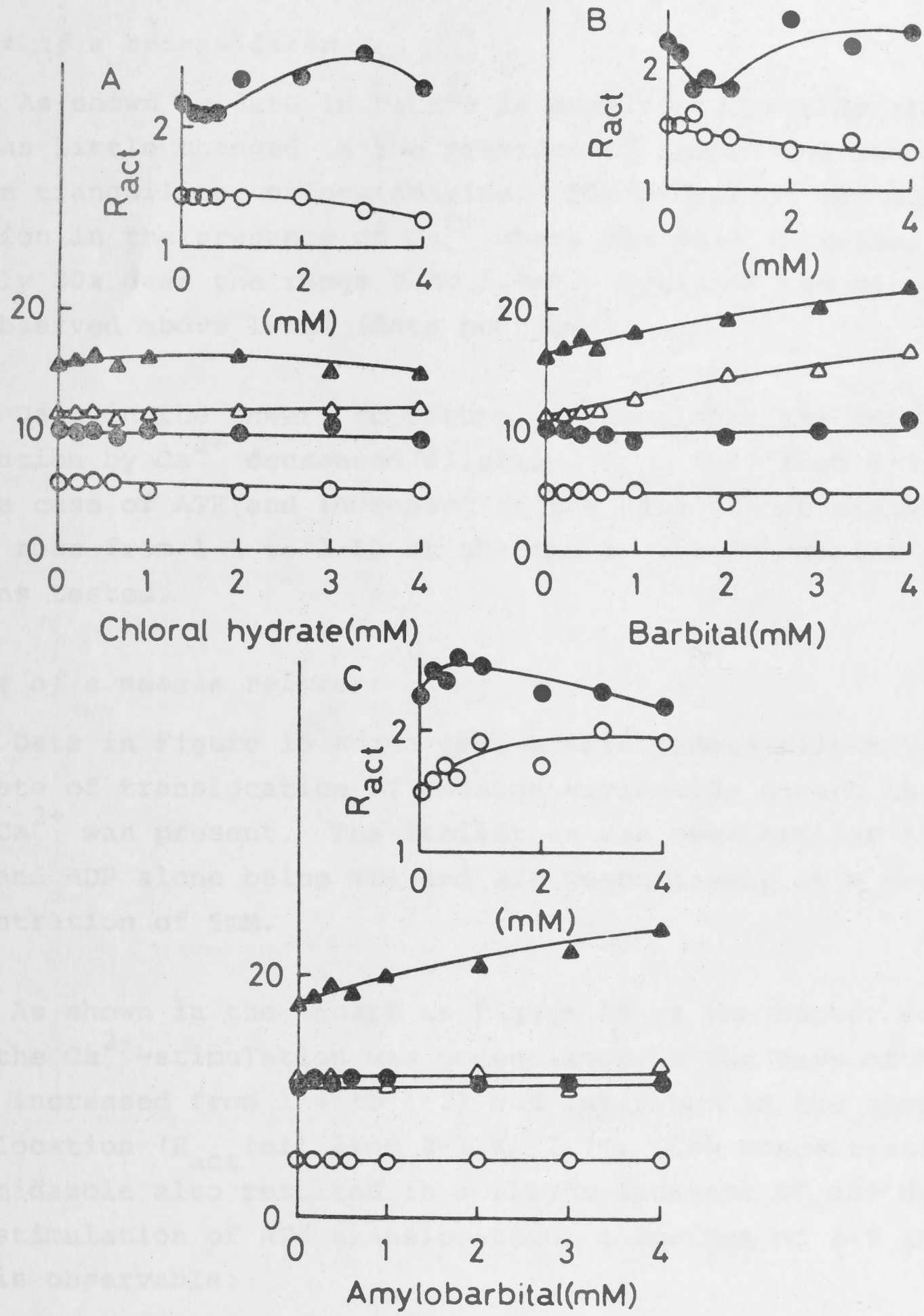


Figure 13. *Effect of hypnotics and sedatives on the translocation of adenine nucleotides.*

Mitochondria were incubated as described in the legend to Figure 2 with 200μM ATP (circles) or ADP (triangles) and various concentrations of effectors in the presence (closed symbols) or absence (open symbols) of 200μM Ca^{2+}. A, Chloral hydrate; B, Barbital; C, Amylobarbital.
Inserts show the relative activation by Ca^{2+} of ATP (●) or ADP (O) translocation.
v refers to the rate of translocation of adenine nucleotide in nmoles/min per mg protein.

Effect of a tranquilizer

As shown by data in Figure 14 adenine nucleotide translocation was little changed in the presence of increasing concentrations of the tranquilizer chlorpromazine. The exception was ADP translocation in the presence of Ca^{2+} where the rate increased approximately 30% over the range 0 to 1.0mM. Lysis of the mitochondria was observed above 1·5mM (data not shown).

Data in the insert to Figure 14 shows that the relative activation by Ca^{2+} decreased slightly (R_{act} fell from 2·3 to 2·1) in the case of ATP and increased in the case of ADP translocation (R_{act} rose from 1·3 to 1·8) at the higher chlorpromazine concentrations tested.

Effect of a muscle relaxant

Data in Figure 15 shows that benzimidazole slightly decreased the rate of translocation of adenine nucleotide except that of ADP when Ca^{2+} was present. The inhibition was greatest for ATP plus Ca^{2+} and ADP alone being 40% and 25% respectively at a drug concentration of 5mM.

As shown in the insert to Figure 15 at the higher concentrations the Ca^{2+}-stimulation was potentiated in the case of ADP (R_{act} increased from 1·4 to 1·7) and inhibited in the case of ATP translocation (R_{act} fell from 2·1 to 1·7). Low concentrations of benzimidazole also resulted in a slight increase of the degree of Ca^{2+}-stimulation of ATP translocation; a maximum of 2·5 in the R_{act} is observable.

Effect of anti-inflammatory drugs

The compounds tested in this grouping were salicylic and acetylsalicylic acids (Figure 16). The latter had no effect on ATP translocation but slightly stimulated that of ADP. The stimulation was relieved at higher concentrations in the presence of Ca^{2+}. Salicylic acid, however, produced entirely different responses. ADP was unaffected except when Ca^{2+} was present when it mimicked the results obtained with acetylsalicylic acid. ATP translocation was stimulated in the absence of Ca^{2+} and slightly inhibited in its presence.

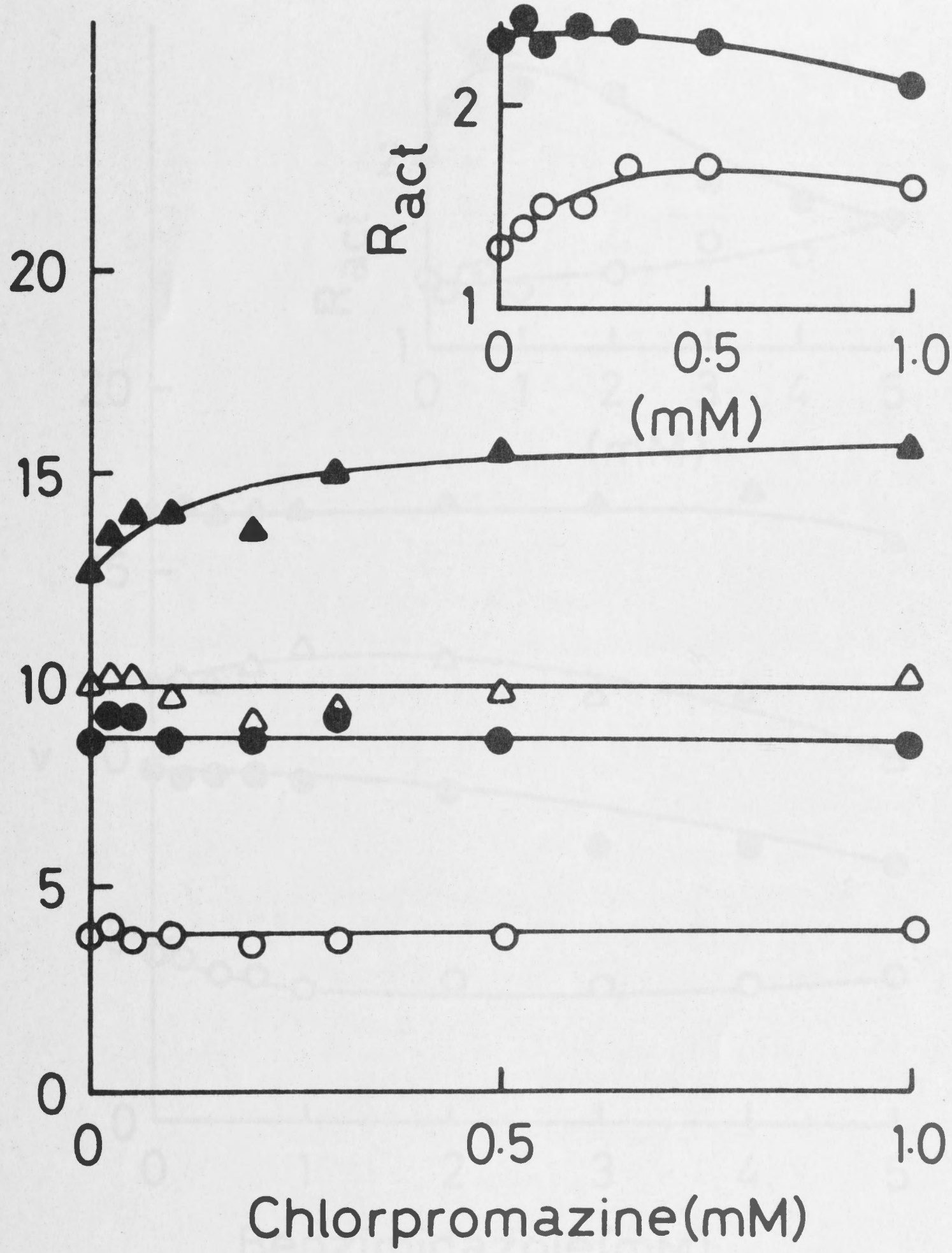


Figure 14. *Effect of chlorpromazine on the translocation of adenine nucleotides.*

Mitochondria were incubated as described in the legend to Figure 2 with 200μM ATP (circles) or ADP (triangles) and various concentrations of chlorpromazine in the presence (closed symbols) or absence (open symbols) of 200μM Ca^{2+}. Inserts show the relative activation by Ca^{2+} of ATP (●) or ADP (O) translocation. v refers to the rate of adenine nucleotide translocation in nmoles/min per mg protein.

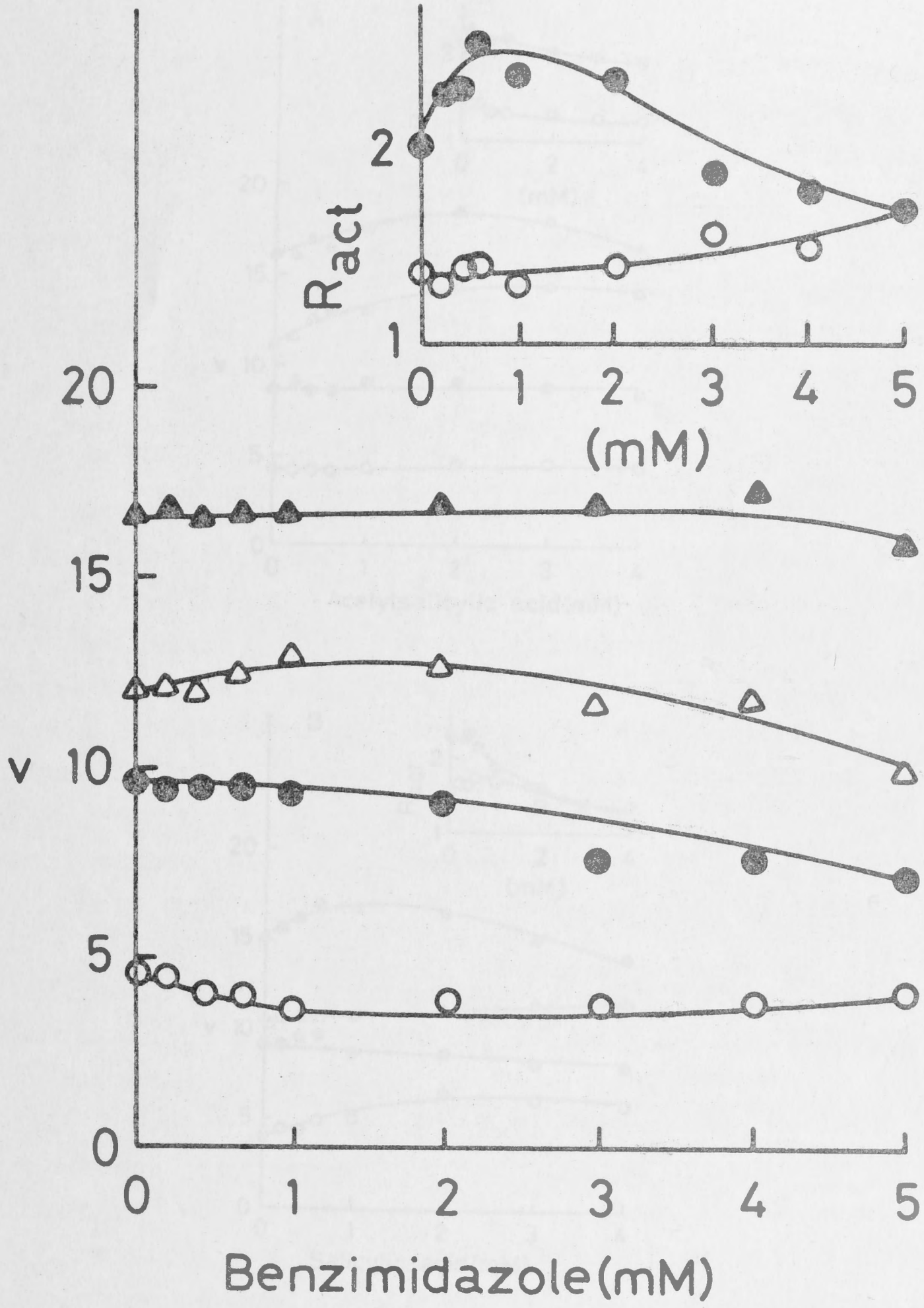


Figure 15. *Effect of benzimidazole on the translocation of adenine nucleotides.*

Mitochondria were incubated as described in the legend to Figure 2 with 200 µM ATP (circles) or ADP (triangles) and various concentrations of benzimidazole in the presence (closed symbols) or absence (open symbols) of 200 µM Ca^{2+}. Inserts show the relative activation by Ca^{2+} of ATP (●) or ADP (O) translocation. v refers to the rate of adenine nucleotide translocation in nmoles/min per mg protein.

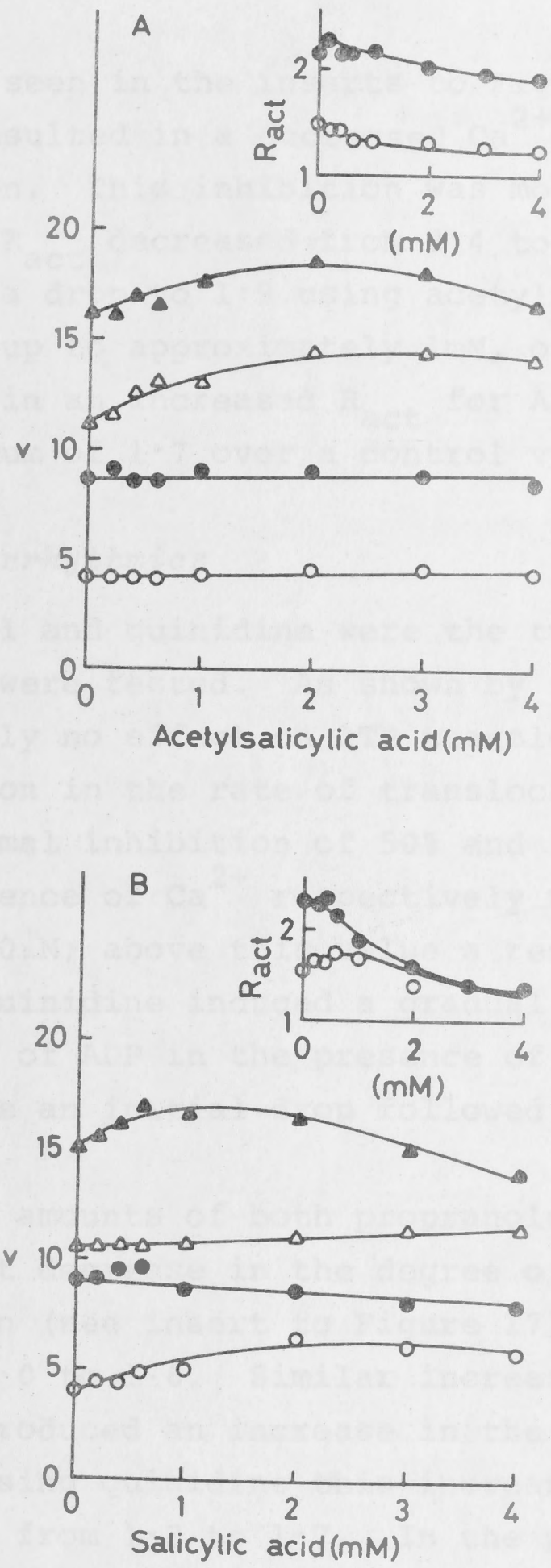


Figure 16. *Effect of anti-inflammatory drugs on the translocation of adenine nucleotides.*

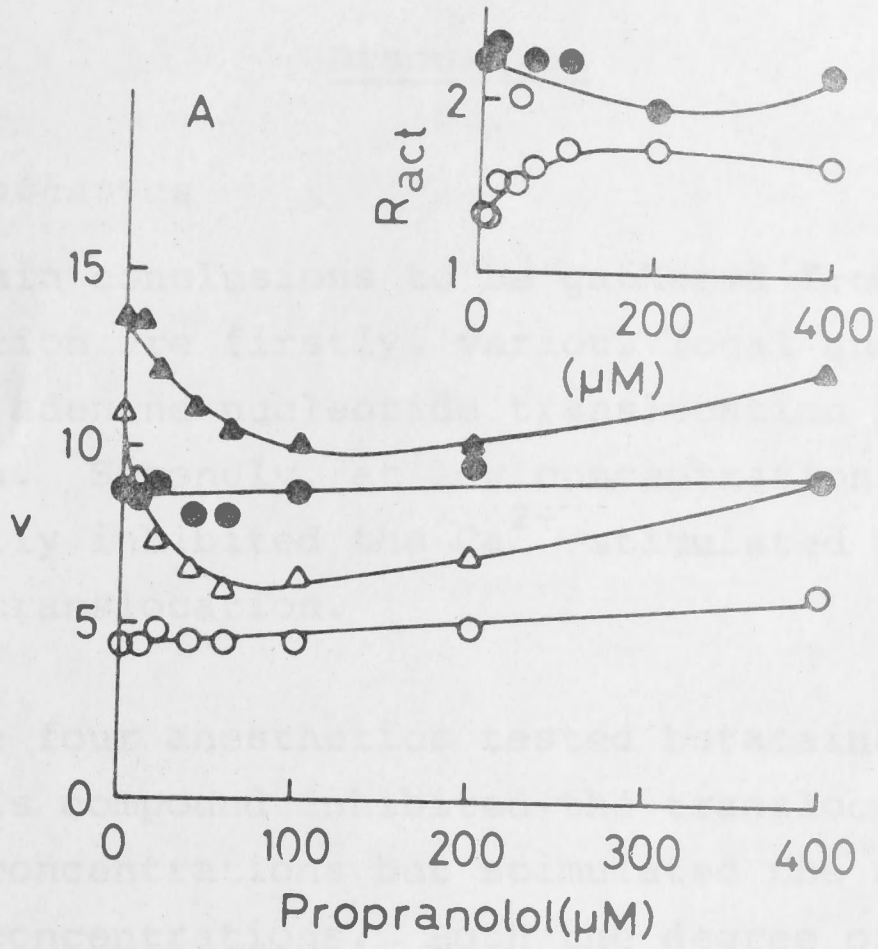
Mitochondria were incubated as described in the legend to Figure 2 with 200μM ATP (circles) or ADP (triangles) and various concentrations of effectors in the presence (closed symbols) or absence (open symbols) of 200μM Ca^{2+}.
A, acetylsalicylic acid; B, salicylic acid.
Inserts show the relative activation by Ca^{2+} of ATP (●) or ADP (○) translocation.
v refers to the rate of adenine nucleotide translocation in nmoles/min per mg protein.

As can be seen in the inserts to Figure 16 high concentrations of both drugs resulted in a decreased Ca^{2+}-stimulation of ATP and ADP translocation. This inhibition was more marked in the case of salicylic acid; R_{act} decreased from 2·4 to 1·2 for ATP translocation compared to a drop to 1·9 using acetylsalicylic acid. Low concentrations, up to approximately 1mM, of salicylic acid did, however, result in an increased R_{act} for ADP translocation; R_{act} achieved a maximum of 1·7 over a control value of 1·4.

Effect of anti-arrhythmics

Propranolol and quinidine were the two representatives of this class that were tested. As shown by the plots of Figure 17 both had virtually no effect on ATP translocation but, however, induced inhibition in the rate of translocation of ADP. Using propranolol maximal inhibition of 50% and 30% for ADP in the presence and absence of Ca^{2+} respectively was observed at approximately 100μM; above this value a restoration of the rate was observed. Quinidine induced a gradual decrease in the rate of translocation of ADP in the presence of Ca^{2+}. In the absence of Ca^{2+} there was an initial drop followed by a plateau.

Increasing amounts of both propranolol and quinidine produced a slight decrease in the degree of stimulation by Ca^{2+} of ATP translocation (see insert to Figure 17). With the former R_{act} fell from 2·0 to 1·6. Similar increases in drug concentrations, however, produced an increase in the stimulation of ADP translocation; using quinidine this increase was approximately 30% with R_{act} rising from 1·3 to 1·7. In the presence of higher propranolol concentrations the potentiation was reversed.



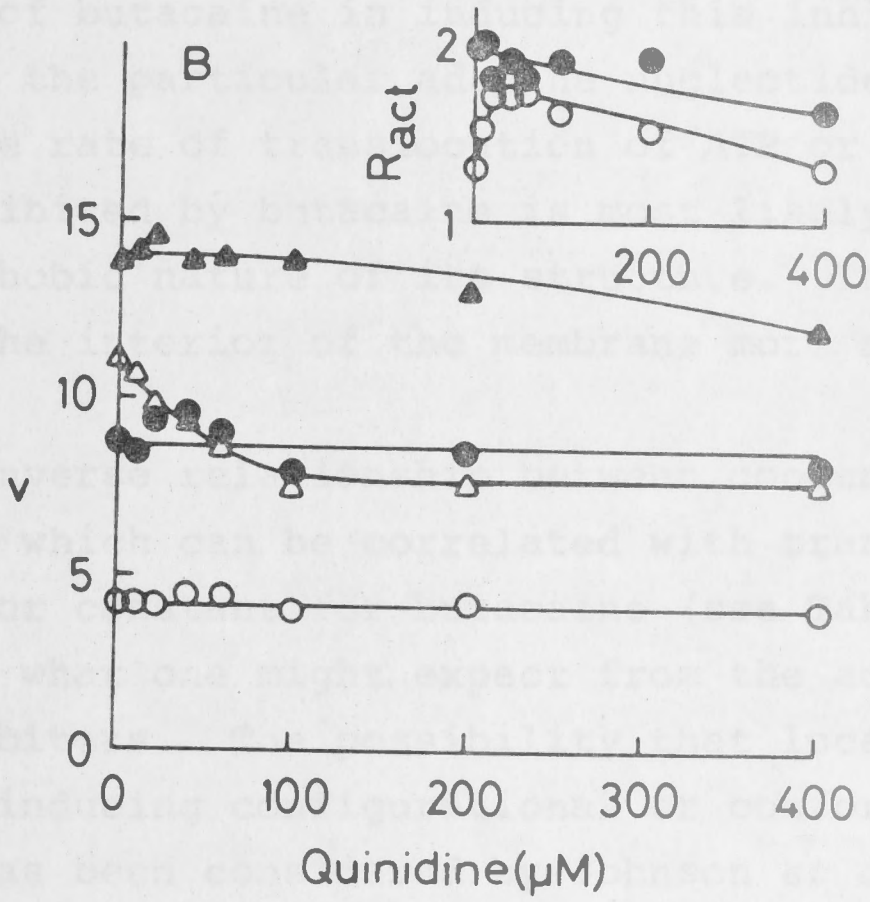


Figure 17. *Effect of anti-arrhythmics on the translocation of adenine nucleotides.*

Mitochondria were incubated as described in the legend to Figure 2 with 200μM ATP (circles) or ADP (triangles) and various concentrations of effectors in the presence (closed symbols) or absence (open symbols) of 200μM Ca^{2+}. A, propranolol; B, quinidine. Inserts show the relative activation by Ca^{2+} of ATP (●) or ADP (○) translocation. v refers to the rate of adenine nucleotide translocation in nmoles/min per mg protein.

Discussion

1 Local Anesthetics

The main conclusions to be gathered from the data presented in this section are firstly, various local anesthetics inhibit the overall adenine nucleotide translocation process in rat liver mitochondria. Secondly, at low concentrations, anesthetics preferentially inhibited the Ca^{2+}-stimulated portion of adenine nucleotide translocation.

Of the four anesthetics tested butacaine was by far the most potent. This compound inhibited the translocation at high adenine nucleotide concentrations but stimulated the system at low adenine nucleotide concentrations. Both the degree of inhibition and the efficiency of butacaine in inducing this inhibition were dependent not only on the particular adenine nucleotide examined but also on the absolute rate of translocation of ATP or ADP. The greater potency exhibited by butacaine is most likely attributable to the more hydrophobic nature of its structure. This would allow it to penetrate the interior of the membrane more readily.

The inverse relationship between concentration of adenine nucleotide, which can be correlated with translocation activity, and inhibitor constant for butacaine (see Tables I and II) is contrary to what one might expect from the action of classical enzyme inhibitors. The possibility that local anesthetics are capable of inducing configurational or conformational changes in membranes has been considered by Johnson *et al* (113). In the light of this theory then the above observations could be explained in the following manner. Interaction of butacaine with the mitochondrial membrane induces a state of the membrane in the vicinity of the translocase such that its mobility and consequently, translocation activity, is decreased. An analogous situation is the effect of lowering the incubation temperature which achieves a similar result.

Under conditions where the translocation rate of adenine nucleotides is high it is suggested that the fluidity of the membrane is an important factor in determining the activity of the translocase. This has been indeed shown for the adenine nucleotide translocase which exhibits a very high temperature dependence as

evidenced by the activation energy of 35Kcal/mole at 4°C (201). By analogy a small change in membrane fluidity induced by a low butacaine concentration would be expected to cause a large change in the translocation rate. Conversely when the translocase is operating at a relatively low rate the fluidity of the membrane would have to be decreased to a large extent to achieve a commensurate decrease in the rate of translocation.

One point which remains unresolved by the above explanation is the observation that the basal rate of translocation after butacaine inhibition is the same for all ATP and all ADP concentrations tested. Thus, under these conditions, the translocation reaction is fully saturated even at 10μM adenine nucleotide. This may be compared to data obtained by Klingenberg *et al* (201) who have obtained, using special conditions of low mitochondrial protein concentrations, a K_m for translocation of approximately 1μM, one tenth that of the lowest concentration we have used. It is not inconceivable then, and this is supported by data provided above that as the butacaine concentration is increased the affinity for adenine nucleotide also increases. To this end ADP which is translocated 2-3 times faster than ATP also has a basal level which is 2-3 times higher than that of ATP (see Figures 4 and 5).

All the local anesthetics tested inhibited the stimulation by Ca^{2+} of ADP and ATP translocation. In general the relative potency of these compounds correlated with their potency on nerve conduction blockage (225). The exception to this was butacaine but in view of the exceptional properties of butacaine with regard to inhibition of the translocation of adenine nucleotide no importance was attached to this discrepancy. In the case of Ca^{2+}-stimulated ADP translocation the concentrations necessary for 50% inhibition were higher than those required for an equivalent inhibition of Ca^{2+}-stimulated ATP translocation. If one assumes that this inhibition is medicated via competition between the local anesthetic and Ca^{2+} binding to the mitochondrial membranes then this difference can be explained in terms of 'free' Ca^{2+} ions available to these sites. Under the conditions employed, i.e. 200μM adenine nucleotide and 200μM Ca^{2+}, one may calculate the 'free' Ca^{2+} concentrations (see refs.231,245) which are 108μM and 178μM in the presence of ATP and ADP respectively.

Low concentrations of anesthetic produced apparently contradictory results in that they potentiated the stimulatory effects of Ca^{2+} on the translocation of ADP but not of ATP. Similar adenine nucleotide specificity has been observed previously (Section C) with respect to La^{3+}, a known Ca^{2+} antagonist, which has been shown to inhibit the stimulation of ATP translocation but, as above, potentiates the stimulation of ADP translocation. There appears to be some type of synergism operative between both La^{3+} on the one hand and Ca^{2+} and local anesthetics and Ca^{2+} on the other resulting in a stabilisation of the mitochondrial membrane which highlights the stimulatory effect of the Ca^{2+}. In relation to this Mela has observed that low concentrations of butacaine stimulate the membrane alkinisation changes associated with Ca^{2+} accumulation by mitochondria (167).

On the basis of these and other results it is suggested that the mechanism by which Ca^{2+} stimulates ATP and ADP translocation differs, although it has previously been shown that the concentrations of 'free' Ca^{2+} required to half-maximally stimulate are equivalent (Section C). A second possibility consistent with this data is that the mechanisms by which ATP and ADP are bound and/or translocated by the mitochondria are not identical. Supporting evidence for this hypothesis comes from several sources: (a) the response of ATP and ADP translocation to increasing amounts of nupercaine, tetracaine and procaine differ markedly (see Figures 2 and 3), and (b) the high apparent K_m for adenine nucleotide decreases in the case of ATP and increases with ADP as substrate in the presence of increasing butacaine concentrations.

Another possibility which is not inconsistent with the above is that there exists in the mitochondrial membrane two types or classes of adenine nucleotide translocase molecules with separate and distinct properties and/or microenvironments which may be specific for ATP or ADP.

Inhibition of the translocase reaction by butacaine exhibits a pH dependence (Figure 7) which cannot be correlated with the proportion of ionised butacaine molecules present in the incubation medium. As local anesthetics are lipid-soluble molecules it was suggested that changes in the membrane-bound phospholipids themselves may be responsible for this pH-dependence. However,

experiments have shown that the pKa's for individual purified phospholipids (88,188,213,226) and indeed whole mitochondrial phospholipids (110) do not coincide with the decreased inhibition by butacaine observed at acid pH. One must then resort to an explanation which relies on a pH-dependent change in the micro-environment of the translocase and/or butacaine binding sites which produces the diminished inhibitory effect of butacaine on the translocation reaction.

In contrast to the effects of pH on the inhibition of adenine nucleotide translocation by butacaine, pH had virtually no effect on the stimulation by butacaine of the translocation at low concentrations of adenine nucleotide (see Table X).

It is difficult to reconcile the two types of response to butacaine, i.e. inhibition and stimulation, with binding of butacaine to a single class of butacaine binding site. Thus it is proposed that butacaine binds to at least two different types of binding sites on the mitochondrial membrane.

Two types of sites which readily come to mind are the negatively charged groups in the membrane, from which local anesthetics displace bound Ca^{2+}, and the lipid phase of the membrane itself which is capable of solubilizing the hydrophobic portion of the drug. Butacaine may also, however, interact with some of the non-polar proteins which are known to comprise a large % of the total membrane protein (29). Little is known concerning local anesthetic binding to mitochondrial membranes although several superficial reports have appeared (83,143,144,223,265). Recently, however, experiments have been reported which indicate that chlorpromazine, promethazine and laurylamine, three membrane-active drugs that possess local anesthetic properties, bind to the mitochondrial membrane at two independent sets of sites (106). Under conditions where adenine nucleotide interaction with the membrane is minimal one of these sites would be favoured and so stimulation would be evident. Increasing concentrations of either adenine nucleotide or butacaine itself would reverse the order of interaction and so the seond site would being to exert its inhibitory effects. Furthermore, it may be that stimulation of

translocation does occur at higher adenine nucleotide concentrations but that, as indicated by data in Tables I and II, this stimulation would require low butacaine concentrations, lower than those tested. This proposition that at least two classes of butacaine binding sites exist is consistent with the observations that,

(a) the inhibition of translocation of high concentrations of adenine nucleotide is only partially inhibited by competition between local anesthetics,

(b) the stimulation of translocation of low concentrations of adenine nucleotide is completely inhibited by competition between local anesthetics,

(c) low pH only partially inhibits the degree of inhibition of adenine nucleotide translocation by butacaine.

Alternatively it is possible that the two effects, stimulation and inhibition, are mediated via the one binding site which has properties such that a slight conformation change in the membrane exerts either a stabilising or 'non-stabilising' effect according to the concentration of adenine nucleotide present. In this regard it is well documented that adenine nucleotide produces conformational changes in the mitochondrial membrane (241,242,265,266) and it has been suggested, as mentioned above, that local anesthetics produce similar effects (113).

Membrane charge seems to be an important factor involved in the ability of butacaine to inhibit adenine nucleotide translocation. In this regard it is the gross membrane charge elicited by 20mM K^+, and not localised positive charge caused by 50μM La^{3+} which is the more important. It has been shown previously that K^+ and Ca^{2+}-mediated stimulation of adenine nucleotide translocation are partially additive indicating different binding sites on the mitochondrial membrane in these phenomena (Section C). Furthermore the Ca^{2+}, but not the K^+ effect is sensitive to low concentrations of La^{3+}. These plus the above observations indicate that the Ca^{2+} binding sites on the mitochondrial membrane are not involved in the inhibitory action of butacaine on the translocation process. It is a possibility then that this effect may be mediated by butacaine interacting with the phospholipid hydrophobic regions of the membrane. Evidence to support this hypothesis comes from the structures of the local anesthetics (Figure 1); of all the compounds tested butacaine is the only one possessing two butyl groups, a property which would make it much more lipid soluble than the rest.

There is considerable evidence that the physiological sites of action of local anesthetics are the membranes of various cells and subcellular organelles (for review see ref.225). Thus it is highly likely that one of the functions of the mitochondria to be effected by anesthetic action *in vivo* is adenine nucleotide translocation. This is especially so for butacaine which has been shown to have such a high affinity for this process. Butacaine itself has not been widely used in recent years as a local anesthetic due to its toxic side effects (151,217). This study indicates that this toxicity is most likely attributable to inhibition of the adenine nucleotide translocase which would very quickly deplete the cell of its content of ATP generated in the mitochondrion by oxidative phosphorylation.

2 Membrane-active agents

The present study establishes that responses of adenine nucleotide translocation to a particular membrane-active drug are not necessarily the same either in the absence or presence of Ca^{2+}. These observations could be explained by the following: (a) the interaction of ATP and ADP with the membrane is governed not only by charge effects but also by the molecular configuration of the adenine nucleotide molecule and/or conformational changes in the membrane induced by the binding of the drug to the membrane components, (b) concomitant with (a) there may be two different binding and/or translocase molecules, one for ATP and one for ADP, having different membrane environments yet still mutually exclusive, and (c) the mechanism by which Ca^{2+} stimulates ATP and ADP translocation are different. This has been suggested from previous experiments performed using lanthanides (see Section C) which have been found to inhibit the Ca^{2+}-stimulated translocation of ATP, but not that of ADP.

Alcohols stabilise and increase the binding of Ca^{2+} to cell plasma membranes (224). By analogy it would be expected that the stimulatory effect of Ca^{2+} on ATP translocation would be either maintained or enhanced. This was not the case in the present system, however, where inhibition occurred at all alcohol concentrations tested. The maxima in the translocation rates in the absence of Ca^{2+} could be explained on the basis of a stabilisation and/or increase in the fluidity of the membrane produced by the alcohol (see ref.225). The fact that these maxima occur only in the absence of Ca^{2+} ions indicates that the process is sensitive to levels of this ion. The effectiveness of tne n-alcohols is as expected if the interactions are mediated via hydrophobic

phospholipid in that the longer chain length alcohols were more potent. This is corroborated by the results obtained with branched-chain alcohols where the decreased hydrophobicity of these isomers resulted in their being less effective at lower concentrations.

Chlorpromazine uncouples oxidative phosphorylation (144) and also inhibits both respiration and ATP-supported Ca^{2+} uptake (248). The effects of this drug are unlike the classical uncouplers in that it also inhibits the binding of heavy metals to these organelles (110,212). These binding sites are protein in nature as opposed to the binding sites for alkali earth metals which have been shown to be lipid (212,213). These facts would then explain the observation that chlorpromazine has no effect on Ca^{2+}-stimulated ATP translocation. One unexplained result is its stimulation of ADP translocation in the presence of Ca^{2+} but this may be related to the different mechanism of stimulation of ATP and ADP (see above).

Although propranolol and quinidine have similar pharmacological properties they exhibited little similarity on adenine nucleotide translocation. Noack and Greef (182) using an indirect method (measurement of oxygen uptake) came to the conclusion that propranolol and quinidine inhibited the translocation of ADP by rat liver mitochondria. Results presented in this section utilizing a more direct technique indicate that this is not true and indeed other workers have localised the inhibition, of propranolol at least, to be between NAD^{+} and flavoprotein in the respiratory chain (218). The lack of a significant inhibitory effect on the Ca^{2+}-stimulated portion of the translocation precludes the binding of these drugs to mitochondrial phospholipid from being important with respect to membrane-bound Ca^{2+}.

Of the remainder of the membrane-active drugs tested none gave any significant indication of inhibition of Ca^{2+}-stimulated ATP translocation with the possible exception of benzimidazole and salicylic acid. A common response to all the above-mentioned drugs was a stimulation of ADP translocation in the presence of Ca^{2+}.

In conclusion Ca^{2+}-stimulated adenine nucleotide translocation does not respond to membrane Ca^{2+} active agents as one would expect by analogy with studies on nerve and other cell membranes (see ref. 225). This is not unexpected when one compares the gross phospholipid content of the two types of membranes e.g. rat liver mitochondria contains 15% cardiolipin and virtually no sphingelomyelin whereas the plasma membrane contains no cardiolipin and 23% spingomyelin (45). Seeman has pointed out that most *in vitro* work on membrane-bound Ca^{2+} has been centred on the phospholipid moiety yet the majority is bound to protein (75,225). In this respect the mitochondria and plasma membranes also exhibit large differences in protein composition as would be expected with two functionally different membranes. Finally it may be stated that although the mitochondrion provides a good model for the study of drug-membrane interactions one must be careful in directly relating observed results to the *in vivo* situation because of the large compositional differences of these two membranes.

SUMMARY

1 Local anesthetics were found to inhibit translocation of ADP and of ATP; butacaine was the most effective with 50% inhibition occurring at 30μM for 200μM ATP and at 10μM for 200μM ADP. The degree of inhibition by butacaine of both adenine nucleotides was dependent on the concentration of adenine nucleotide present; with low concentrations of adenine nucleotide low concentrations of butacaine-stimulated translocation, but at high concentrations (greater than 50μM) low concentrations of butacaine inhibited translocation. Butacaine increased the affinity of the translocase for ATP to a value which approached that of ADP.

2 Higher concentrations of nupercaine and of tetracaine were required to inhibit translocation of both nucleotides; 50% inhibition of ATP translocation occurred at concentrations of 0·5mM and 0·8mM of these compounds, respectively. Inhibition of ADP translocation by nupercaine and tetracaine was more complex than that of ATP; at very low concentrations (less than 250μM) inhibition ensured followed by a return to almost original rates at 1mM. At higher concentrations inhibition of ADP translocation resulted.

3 That portion of ATP translocation stimulated by Ca^{2+} was preferentially inhibited by each of the local anesthetics tested. In contrast, inhibition by the anesthetics of ADP translocation, was prevented by low concentrations of Ca^{2+}.

4 The data provide further support for the hypothesis that lipid-protein interactions are important determinants in the activity of the adenine nucleotide translocase in mitochondria.

5 Other local anesthetics, low pH and K^{+} ions but not La^{3+} ions decreased the ability of butacaine to inhibit adenine nucleotide translocation. In contrast, at a low ATP concentration the stimulation of the rate of translocation was lost in the presence of other local anesthetics but not under conditions of low pH.

6 Low concentrations of butacaine greatly increased the affinity of the translocase for ATP but only slightly increased that for ADP (low K_m). Concomitantly the high K_m increased in the case of ADP and decreased in the case of ATP.

7 Aliphatic alcohols overall decrease the activity of the adenine nucleotide translocase. This inhibition is preceded by a maximum in the rate of translocation when Ca^{2+} is not present. Potency of the alcohols is directly related to the number of carbon atoms and consequently the hydrophobicity.

8 Membrane-active agents produce different responses of ATP and ADP translocation both in the absence and presence of Ca^{2+}. No significant correlation was observed between drugs which produce similar pharmacological actions and their effects on adenine nucleotide translocation.

9 It is concluded that direct comparisons cannot be made between the effect of membrane-active agents on mitochondrial membranes and membrane-active agents on nerve or any other membrane because of the large differences in both protein and lipid composition.

SECTION E: PHOSPHOLIPIDS AND ADENINE NUCLEOTIDE TRANSLOCATION

Introduction

Results presented in the previous section (D) indicated that the activity of the adenine-nucleotide translocase can be modified by a number of membrane-active agents, the most potent of which is butacaine, which have been shown to interact with the phospholipid component of the membrane (see ref. 225). The majority of these compounds also inhibit the Ca^{2+}-stimulated portion of the translocation. The precise molecular events involved in these two processes, *viz*, membrane-active drug effects and Ca^{2+}-stimulation, have yet to be determined but charge effects and/or conformational changes in the lipid (see refs. 24,89,183) or protein moiety (see refs. 103,131,178,179) of the membrane produced by binding of the effectors are probably involved. This study was extended by examining the effect of controlled lipid depletion of the mitochondrial membranes on the activity of the translocator in the absence and presence of various effector agents including Ca^{2+}.

Many biomembrane-associated reactions are now known to exhibit maximal activity only in the presence of specific phospholipids (see ref. 47 for review). Various techniques have been used to indicate these requirements including (a) extraction of lipids by aqueous acetone and other organic solvents (e.g. 21,46,71), (b) extraction of phospholipids by the action of phospholipase A, C or D (e.g. 65,72,186), (c) solubilisation by detergents (204,244) and (d) the effect of phospholipid oxidation using ascorbic acid or cysteine (211). Definitive proof of lipid requirement, however, requires that three criteria be fulfilled (71): (a) actual physical removal of the lipid, (b) a correlation between removal of the lipid and loss of enzyme activity and (c) a correlation between restoration of the activity and rebinding of the lipid. Thus, as Colemen (47) has pointed out, loss of activity following lipid depletion is not sufficient grounds on its own to establish a lipid dependency. This inactivation may also be due to (a) specific denaturation, unconnected with lipid (this does not apply to phospholipase depletion), (b) inhibitory action of the products of the modification, and (c) direct inhibitory actions of the reagents or their contaminants (e.g. proteases).

Specificity for particular phospholipids has been investigated using phospholipases which hydrolyse particular phospholipids (e.g. 6,81) and/or by adding individual phospholipids back to delipidated preparations (e.g. 21,46,65,71,72,186,204,244). Using the latter technique it has been found that in some cases lyso derivatives are required for maximal activity (e.g. 63,162). This observation may explain the presence of these compounds, albeit in small amounts, in biological membranes (45).

The present studies were performed using purified phospholipase A from porcine pancreas and *Crotalus adamanteus* venom in an effort to also determine the phospholipid specificity in the translocation of ATP and ADP. The former of these two enzymes hydrolyses phospholipids in the order cardiolipin > phosphatidylethanolamine >> phosphatidylcholine (55) whilst the latter enzyme hydrolyses phospholipids in the order phosphatidylcholine = phosphatidylethanolamine; little hydrolysis of cardiolipin occurs (268). Both enzymes are type A_2 phospholipases in that they cleave the fatty acid at position 2 on the phospholipid, i.e. the fatty acid attached to the middle carbon atom of the glycerol backbone.

The data in this section indicate that the translocation of adenine nucleotides themselves in the absence of effectors is dependent on specific phospholipids. Further evidence for the involvement of phospholipids in the stimulation by Ca^{2+} and of CCCP of ATP translocation is also presented. The effect of phospholipid depletion on the binding of adenine nucleotides is also documented.

Results

Specificity of pancreatic and venom phospholipases A for mitochondrial phospholipids

The extent to which mitochondrial phospholipids are hydrolysed by the pancreatic and venom phospholipases A was investigated after labelling of the mitochondrial phospholipids with ^{32}[P] phosphate (see Experimental). The results from one such experiment are shown in Figure 1. The action of the two phospholipases is clearly different, both with regard to substrate specificity and to the shape of the hydrolysis curves obtained. The pancreatic phospholipase (Figure 1a) shows a distinct specificity of the order cardiolipin > phosphatidylethanolamine >> phosphatidylcholine and is consistent with that reported elsewhere (55). Of particular relevance to the work to be described in this section is the extent of phospholipid breakdown in the first 5 minutes of the incubation with the phospholipases. During this time some 40% of the cardiolipin and about 30% of the phosphatidylethanolamine is hydrolysed by the pancreatic phospholipase. However, only a very small proportion of the phosphatidylcholine (about 5%) is hydrolysed in the same time interval.

The venom phospholipase on the other hand exhibits a different phospholipid specificity to the pancreatic enzyme (Figure 1b). Here phosphatidylethanolamine and phosphatidylcholine are each hydrolysed to similar extents whereas cardiolipin is resistant to the action of this enzyme. In the initial 5 minutes about 30% of each phospholipid is hydrolysed.

A complex multi-phasic time course is obtained with pancreatic phospholipase, particularly for its preferred substrate, cardiolipin. The venom enzyme in contrast exhibits pseudo first order reaction kinetics in its hydrolysis of the mitochondrial phospholipids. The maximal rate of hydrolysis of phospholipids with pancreatic phospholipase occurs at approximately 5 minutes. This is also the point of time where the mitochondria exhibit maximal swelling as revealed by optical density changes at 520 nm.

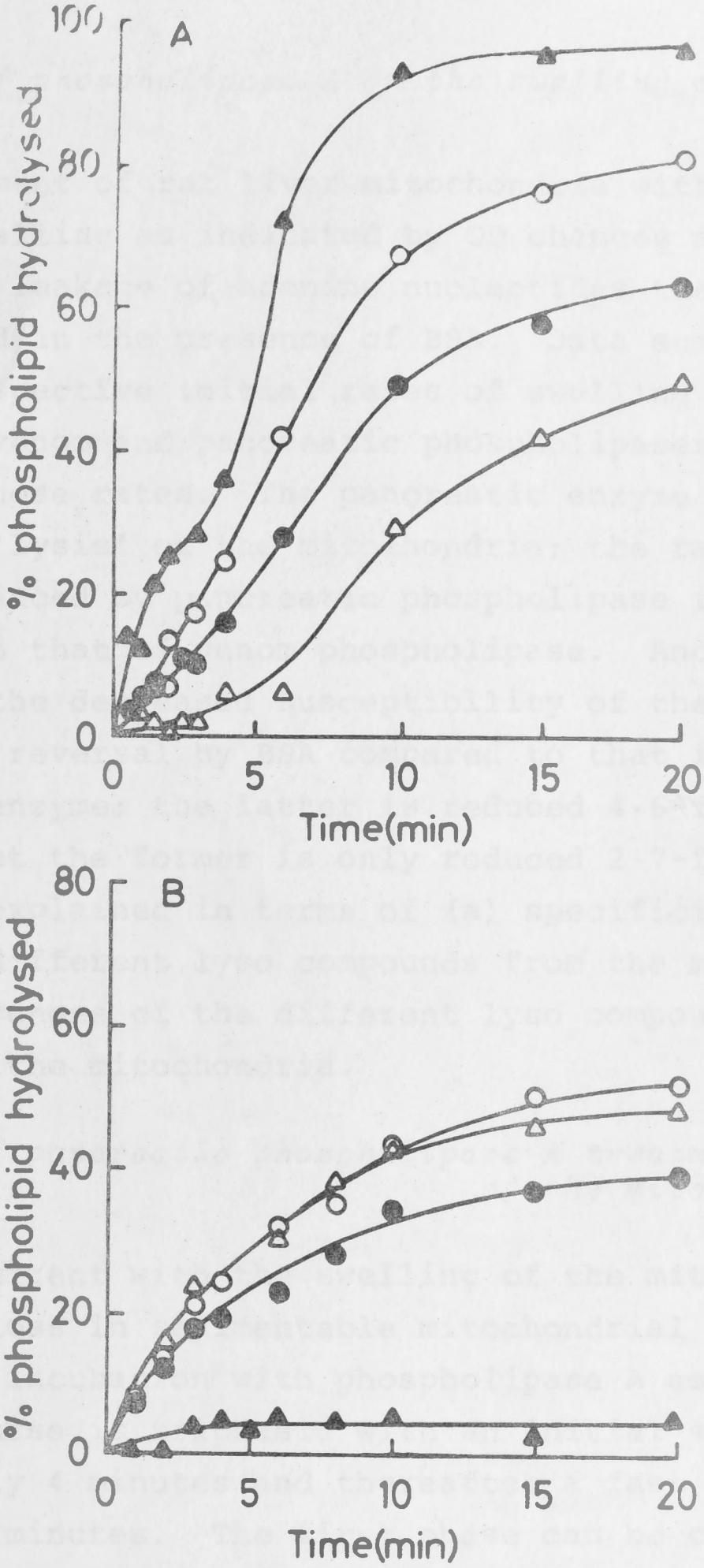


Figure 1. *Ability of phospholipase A to hydrolyse individual mitochondrial phospholipids.*

Mitochondria prelabelled *in vivo* with [^{32}P] inorganic phosphate were incubated with phospholipase as described in Section B in a total volume of 8.0 ml. Samples (0.5 ml) were taken for phospholipid analysis at the times indicated. A, Pancreatic phospholipase A; B, Venom phospholipase A. ▲ ,cardiolipin; ○ , phosphatidylethanolamine; △ ,phosphatidylcholine; ● ,total phospholipid.

Influence of phospholipase A on the swelling of mitochondria

Treatment of rat liver mitochondria with phospholipase A leads to swelling as indicated by OD changes at 520nM (Figure 2). As with the leakage of adenine nucleotides the rate of swelling is decreased in the presence of BSA. Data summarised in Table I show the respective initial rates of swelling of the mitochondria induced by venom and pancreatic phospholipases and also the effect of BSA on these rates. The pancreatic enzyme is the most efficient in causing 'lysis' of the mitochondria; the rate of decrease of the O.D. induced by pancreatic phospholipase is approximately 50% greater than that of venom phospholipase. Another interesting feature is the decreased susceptibility of the venom enzyme-induced swelling to reversal by BSA compared to that induced by the pancreatic enzyme; the latter is reduced 4·6-fold in the presence of BSA whilst the former is only reduced 2·7-fold. This observation can be explained in terms of (a) specificity of BSA for extracting different lyso compounds from the membranes, and/or (b) effectiveness of the different lyso compounds in inducing swelling of the mitochondria.

Influence of pancreatic phospholipase A treatment on the structure of the mitochondrial membranes

Concomitant with the swelling of the mitochondria there also occurred a loss in sedimentable mitochondrial protein with increasing time of incubation with phospholipase A as shown in Figure 3. The time-course is triphasic with an initial slow phase up to approximately 4 minutes and thereafter a fast decline to a plateau at about 15 minutes. The first phase can be correlated with the loss of the outer mitochondrial membrane whilst the second phase is due to the loss of the soluble matrix protein and some inner membrane protein (see ref. 6) after this particular membrane has been lysed.

Influence of pancreatic and venom phospholipases A on the leakage of adenine nucleotides from mitochondria

In order to measure the absolute rate of translocation of adenine nucleotides across the inner mitochondrial membrane, it is necessary to first determine the concentration of endogenous adenine nucleotides contained within the mitochondria. Thus it

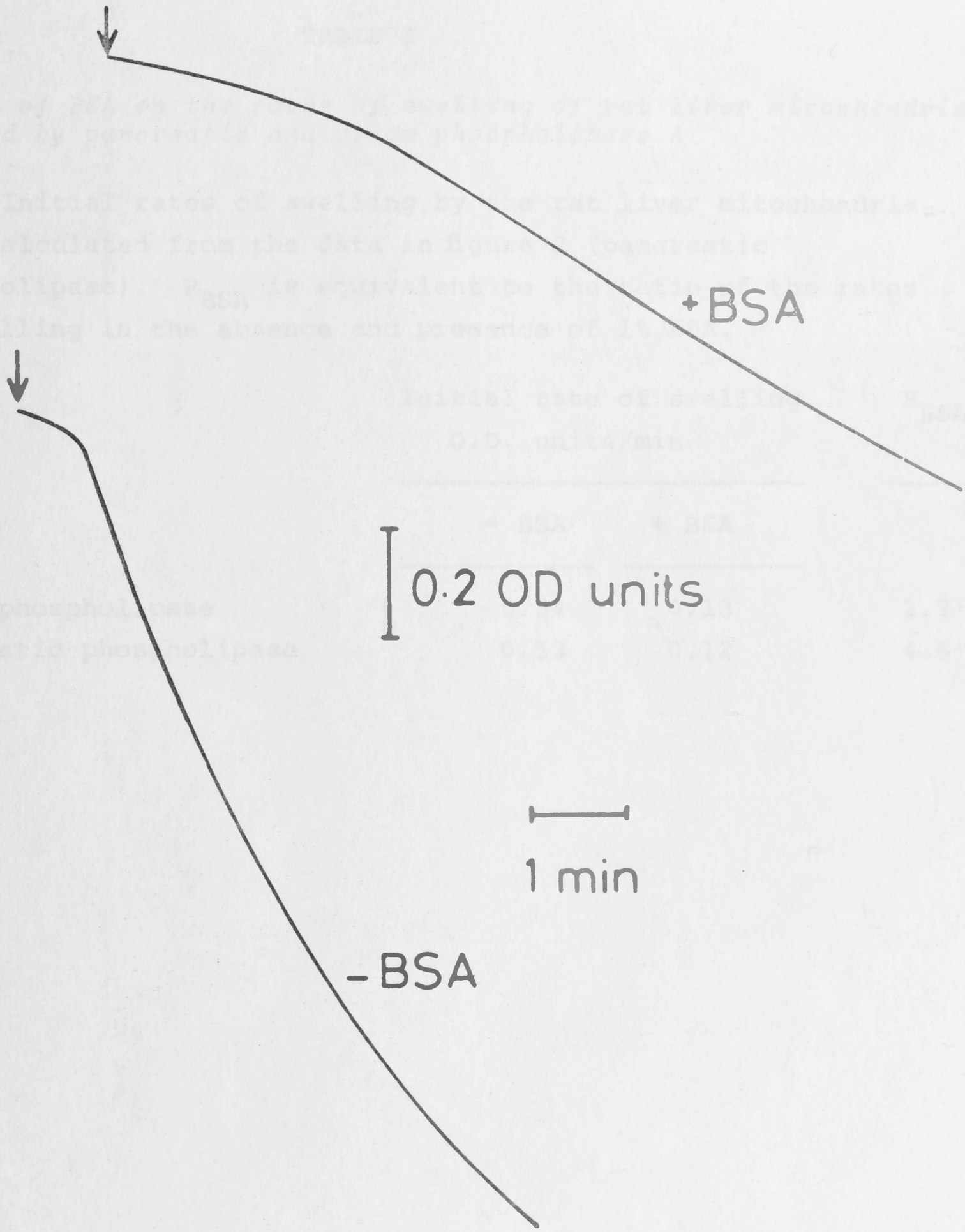


Figure 2. *Pancreatic phospholipase A induced swelling of rat liver mitochondria.*

Mitochondria were incubated as in Section B (except that the protein concentration was 2mg per ml) in spectrophotometer cuvettes at a temperature of 25°C. Swelling was measured by the decrease in O.D. at 520 nm after the addition of pancreatic phospholipase A at the point indicated, either in the presence or absence of 1% BSA.

TABLE I

Effect of BSA on the rates of swelling of rat liver mitochondria induced by pancreatic and venom phospholipase A

Initial rates of swelling by the rat liver mitochondria were calculated from the data in figure 2 (pancreatic phospholipase). R_{BSA} is equivalent to the ratio of the rates of swelling in the absence and presence of 1% BSA.

	Initial rate of swelling O.D. units/min		R_{BSA}
	- BSA	+ BSA	
venom phospholipase	0.34	0.13	2.7
pancreatic phospholipase	0.53	0.12	4.6

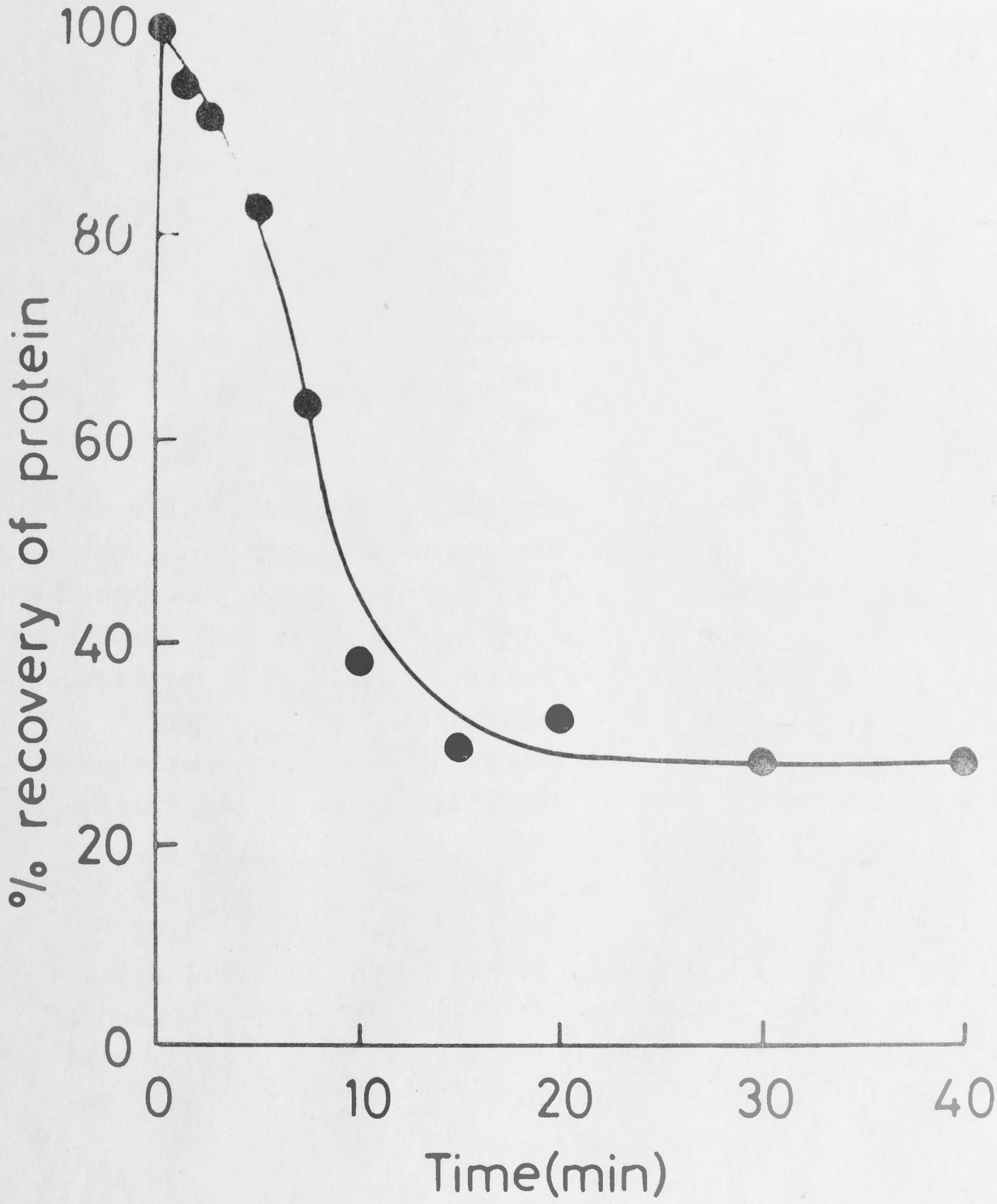


Figure 3. *Recovery of mitochondrial membrane protein after pancreatic phospholipase A treatment*

Mitochondria were incubated as in Section B with pancreatic phospholipase A in the presence of 1% BSA in a total volume of 12ml. At the times indicated 1ml of the mitochondrial suspension (10mg of protein original) was removed, added to 5ml of a solution containing 200mM sucrose, 2mM HEPES, 0.5mM EGTA and 0.5% BSA pH 7.4 and centrifuged for 10 minutes at g values varying between 8,000 (zero time) and 40,000 (15 minutes plus). The mitochondrial pellet was resuspended in 1ml of distilled water containing 0.5% sodium deoxycholate and the protein determined by the biuret method.

Figure 4. Electron-micrographs of pancreatic phospholipase A-treated rat liver mitochondria.

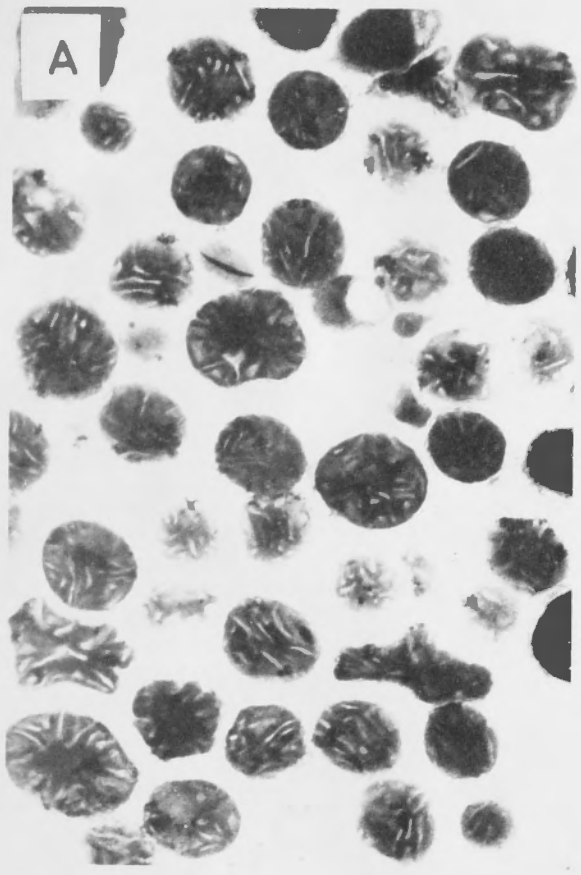
Mitochondria were incubated as described in Section B with pancreatic phospholipase A in the presence of 1% BSA in a total volume of 6ml. At the times indicated 0.5ml of the mitochondrial suspension (5mg of protein) was removed and the samples treated as described in the Experimental section.

Plate (a). Control mitochondria. Outer membrane and the heavily-stained irregular folds of the cristae are quite evident.

Plate (b). 1 minute of treatment. Varying degrees of breakdown in cristal structure has occurred. Approximately 50% of organelles are still 'intact'.

Plate (c). 2½ minutes of treatment. Proportion of swollen mitochondria has greatly increased. Outer membrane seems to be in a highly convoluted state. Some vesicular structures are present.

Plate (d). 4 minutes of treatment. Swelling of the mitochondria is virtually complete. Little breakdown of inner membrane is evident. This is the point in time where the maximal rate of swelling occurs as evidenced by O.D. changes at 520nm.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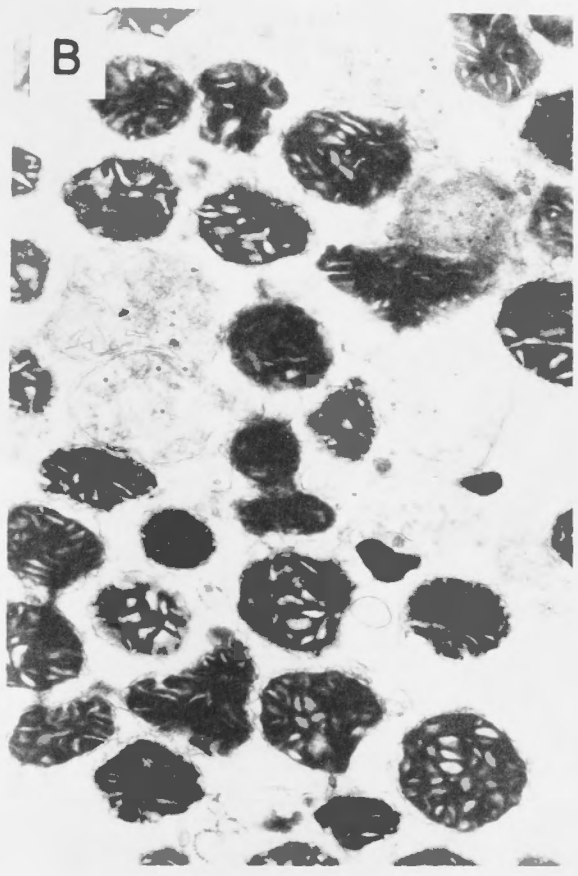
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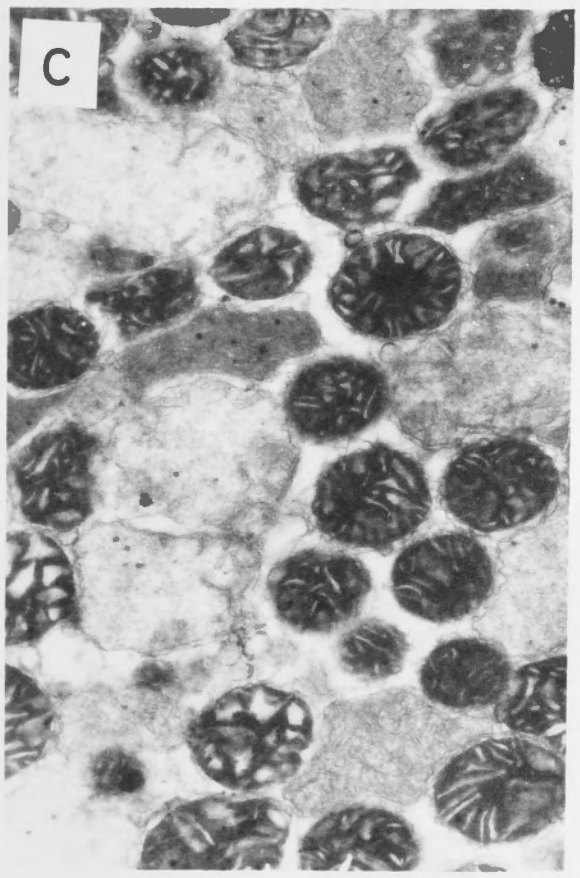
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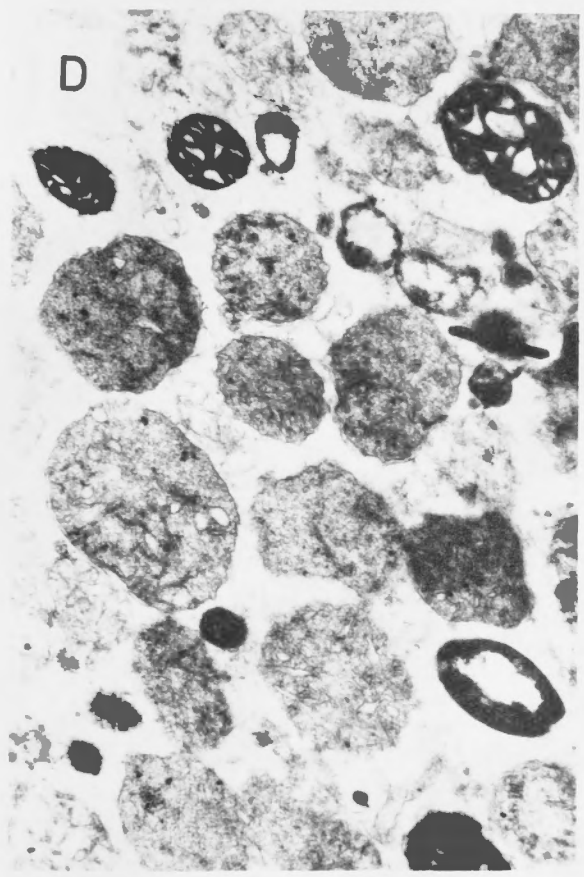
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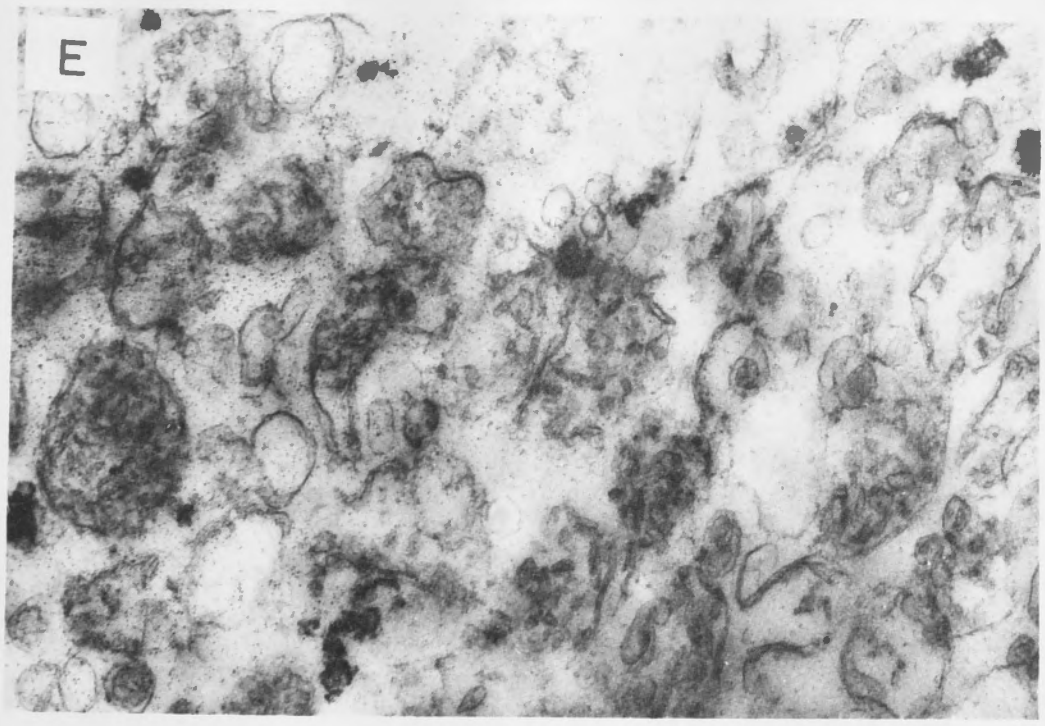
Figure 4. continued

Plate (e). 6 minutes of treatment. All mitochondria have been swollen. In a large per cent of the cases the inner membrane is still intact.
Plate (f). 10 minutes of treatment. Very small proportion of the mitochondrial membrane fragments still retain an intact inner membrane. The large-scale formation of vesicles has occurred.
Plate (g). 15 minutes of treatment. Field consists entirely of diffuse vesicles of varying sizes and shapes.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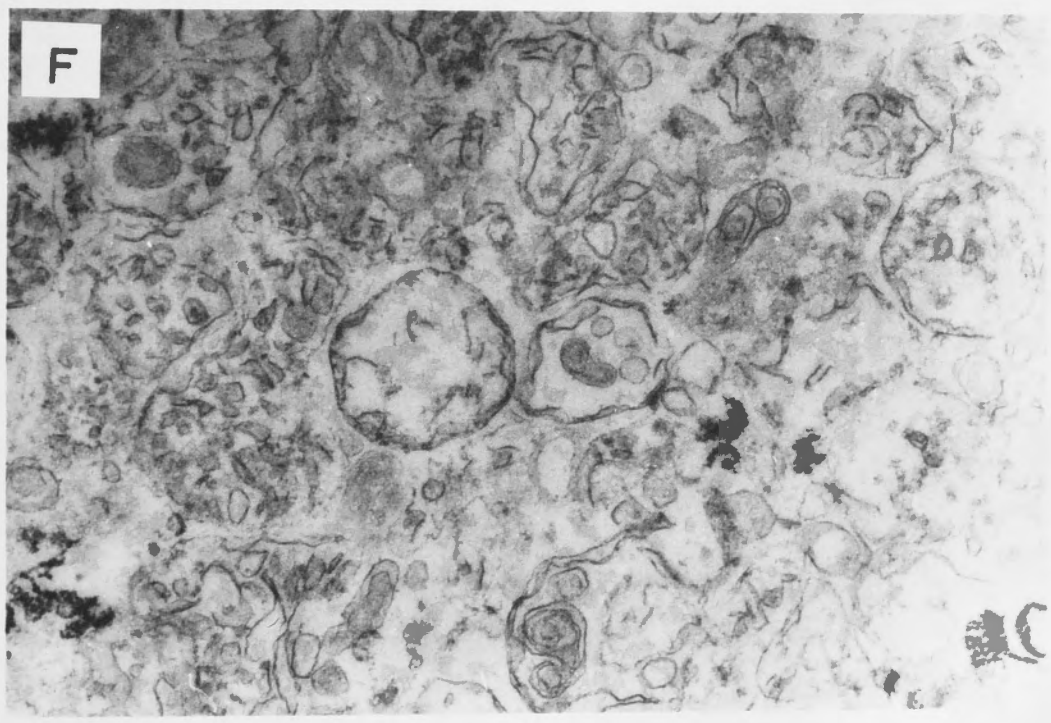
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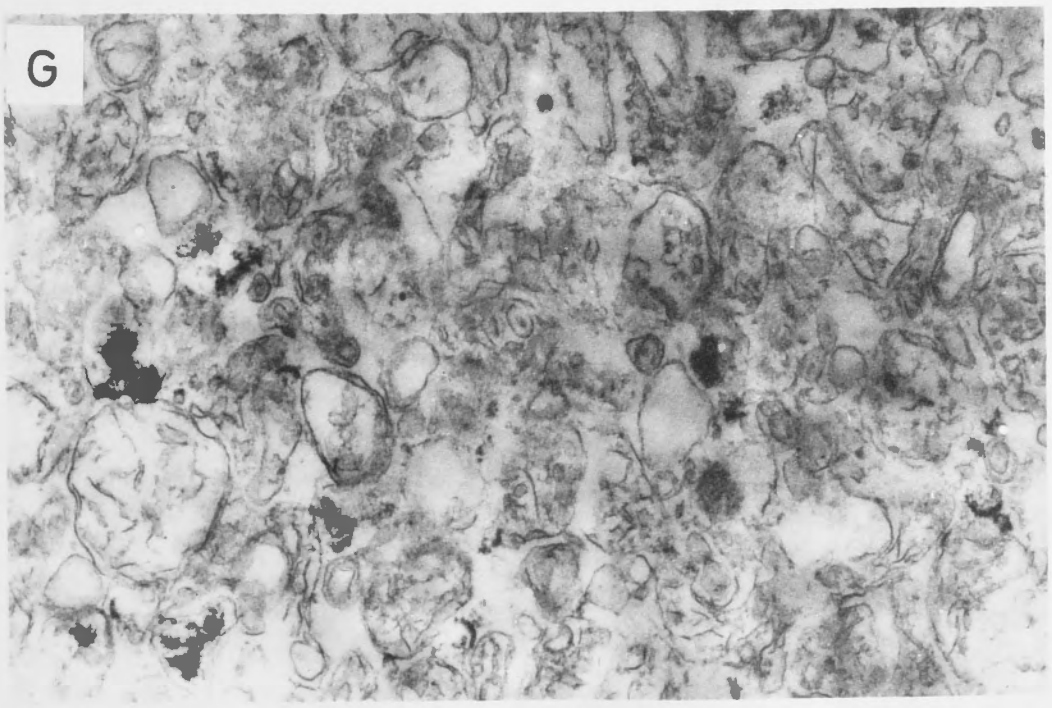
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was necessary at the outset of this study to determine the extent to which endogenous adenine nucleotides leaked from the mitochondria following treatment of the mitochondria with the two phospholipases.

Data in Figure 5 show the loss of intra-mitochondrial adenine nucleotides after various times of incubation with both the pancreatic and venom phospholipase in the presence of 1 mM Ca^{2+}. Several features of the data are noteworthy. Firstly, the pancreatic enzyme is considerably more potent in lysing the organelles and thus promoting adenine nucleotide leakage. The half-times for leakage are approximately one and 3 minutes respectively. Secondly, when 1% BSA is present in the reaction medium with the phospholipases, a time lag is seen in each case which initially reduces the extent of leakage. Under these circumstances the half-times for leakage are about 4 and 6 minutes for pancreatic and venom phospholipases respectively. The third point is that at longer times of incubation the presence of the BSA induces the reverse situation: i.e., the BSA stimulates the loss of adenine nucleotides. In control experiments it was shown that phospholipase-induced leakage of adenine nucleotides did not occur unless 1 mM Ca^{2+} was also present. In all further experiments to be described 1% BSA was present in the incubation medium during the time the mitochondria were treated with phospholipase.

Ability of EGTA to inhibit leakage of adenine nucleotides from mitochondria treated with phospolipases

Both pancreatic and venom phospholipases have a specific requirement for Ca^{2+} and certain other divalent cations (55,268). Therefore it would be expected that lowering the concentration of the available Ca^{2+} in the medium would reduce the ability of the phospholipase to hydrolyse mitochondrial phospholipids and thus prevent release of adenine nucleotides from the mitochondria. As shown in Figure 6 the addition of a four-fold excess of EGTA to mitochondria incubated in the presence of pancreatic phospholipase inhibits the release of endogenous adenine nucleotides. The inhibition is more marked at the shorter times of incubation (about 2 minutes) where the degree of hydrolysis of membrane phospholipids would not be critical with respect to membrane permeability.

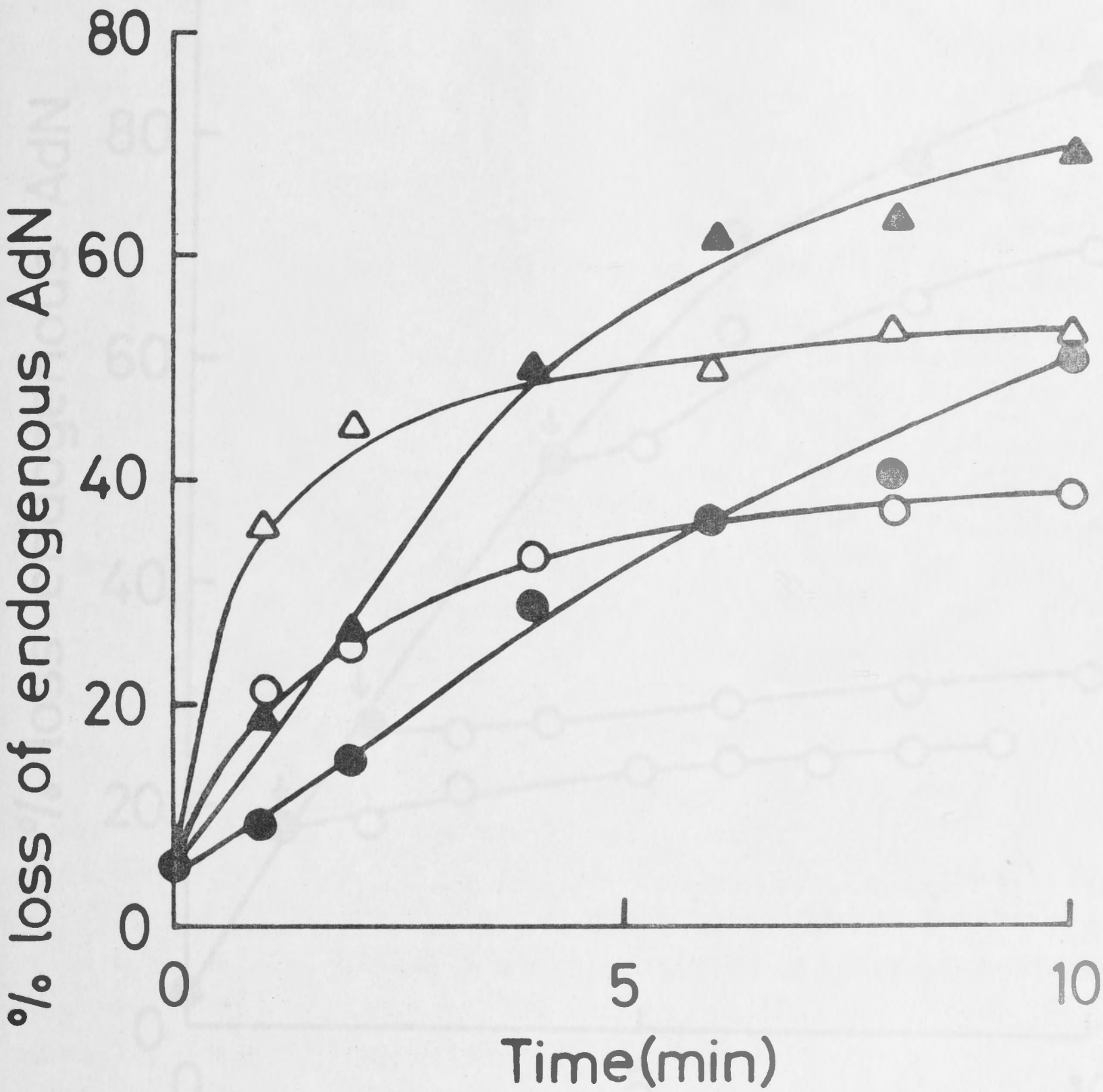

Figure 5. *Effect of time of treatment with phospholipase on loss of endogenous mitochondrial adenine nucleotides.*

Mitochondria prelabelled with [^{3}H] ATP were incubated with phospholipase in the absence (open symbols) or presence (closed symbols) of BSA (1%) as described in Section B in a total volume of 2.0 ml. At the times indicated 0.1 ml of the suspension was removed and centrifuged in an Eppendorf microfuge for 2.5 min. Portions(50 µl) of the supernatant solution were counted to determine the release of radioactivity from the mitochondria.

△ ▲ , pancreatic phospholipase treated mitochondria.

○ ● , venom phospholipase treated mitochondria.

AdN = adenine nucleotide.

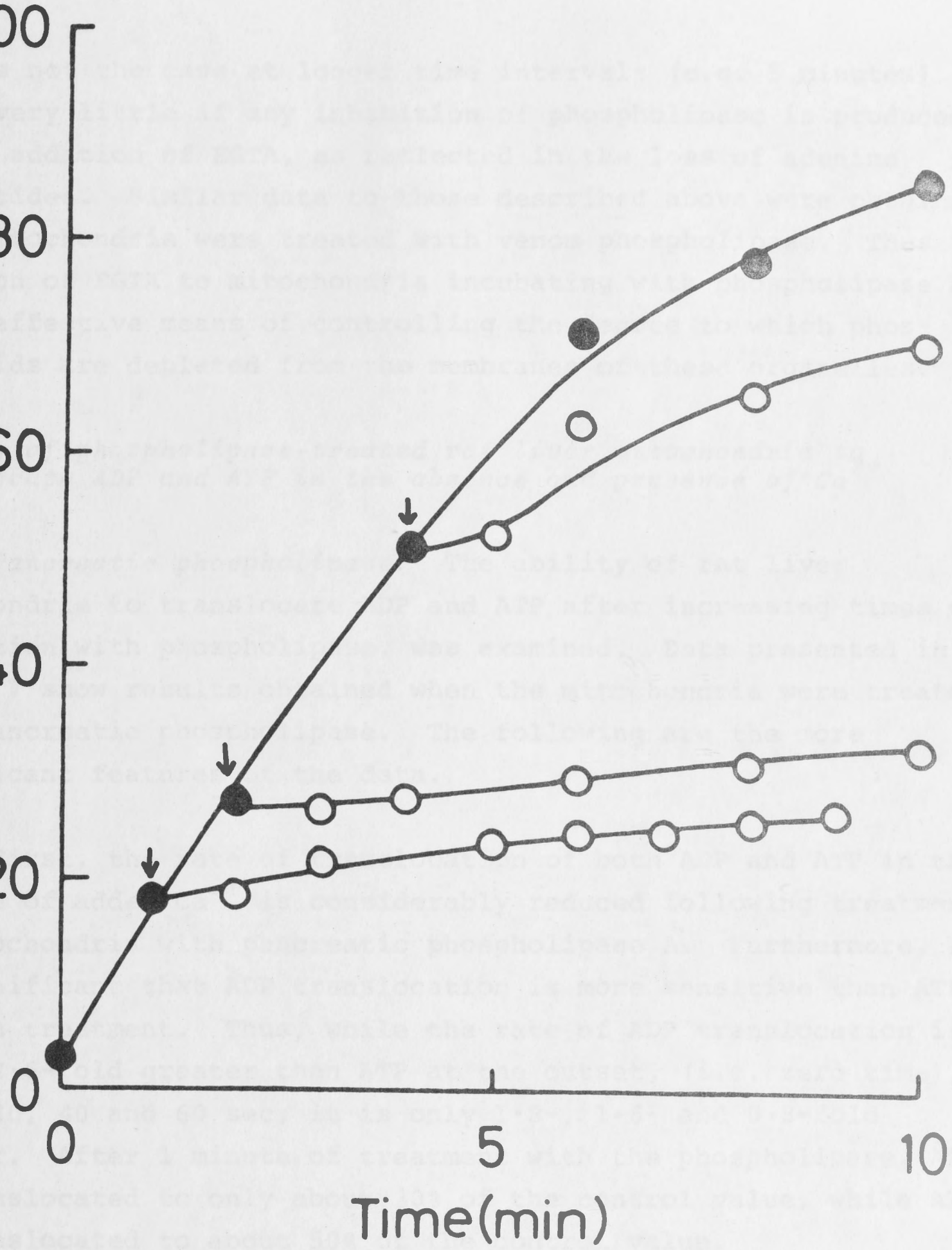

Figure 6. *Effect of EGTA addition on the rate of leakage of adenine nucleotides induced by pancreatic phospholipase A.*

Experimental conditions were as described in Figure 5 except that a separate incubation system was used following the addition of 2mM EGTA (indicated by an arrow) at the times indicated. AdN = adenine nucleotide.

This is not the case at longer time intervals (e.g. 5 minutes) where very little if any inhibition of phospholipase is produced by the addition of EGTA, as reflected in the loss of adenine nucleotides. Similar data to those described above were obtained when mitochondria were treated with venom phospholipase. Thus addition of EGTA to mitochondria incubating with phospholipase A is an effective means of controlling the degree to which phospholipids are depleted from the membranes of these organelles.

Ability of phospholipase-treated rat liver mitochondria to translocate ADP and ATP in the absence and presence of Ca^{2+}

Pancreatic phospholipase. The ability of rat liver mitochondria to translocate ADP and ATP after increasing times of incubation with phospholipase, was examined. Data presented in Figure 7 show results obtained when the mitochondria were treated with pancreatic phospholipase. The following are the more significant features of the data.

First, the rate of translocation of both ADP and ATP in the absence of added Ca^{2+} is considerably reduced following treatment of mitochondria with pancreatic phospholipase A. Furthermore, it is significant that ADP translocation is more sensitive than ATP to such treatment. Thus, while the rate of ADP translocation is about 2·6-fold greater than ATP at the outset, (i.e. zero time), after 20, 40 and 60 sec, it is only 1·8-, 1·6- and 0·8-fold greater. After 1 minute of treatment with the phospholipase, ADP is translocated to only about 30% of the control value, while ATP is translocated to about 50% of the control value.

The other feature of Figure 7 relates to the influence of Ca^{2+} on the translocation of ADP and ATP. It has previously been shown that Ca^{2+} stimulates ATP translocation to a greater degree than ADP translocation (Section C). This is seen also in the data contained in Figure 7 where (at zero time) ATP translocation is enhanced about 2-fold and ADP about 1·3-fold. The ability of Ca^{2+} to stimulate ATP translocation is diminished after treatment of the mitochondria with phospholipase. Following 20, 40 and 60 secs of treatment, the degree of stimulation is reduced to 1·8, 1·7 and 1·6-fold respectively. After 3 minutes of treatment, Ca^{2+} is no longer able to stimulate ATP translocation (see insert to Figure 7).

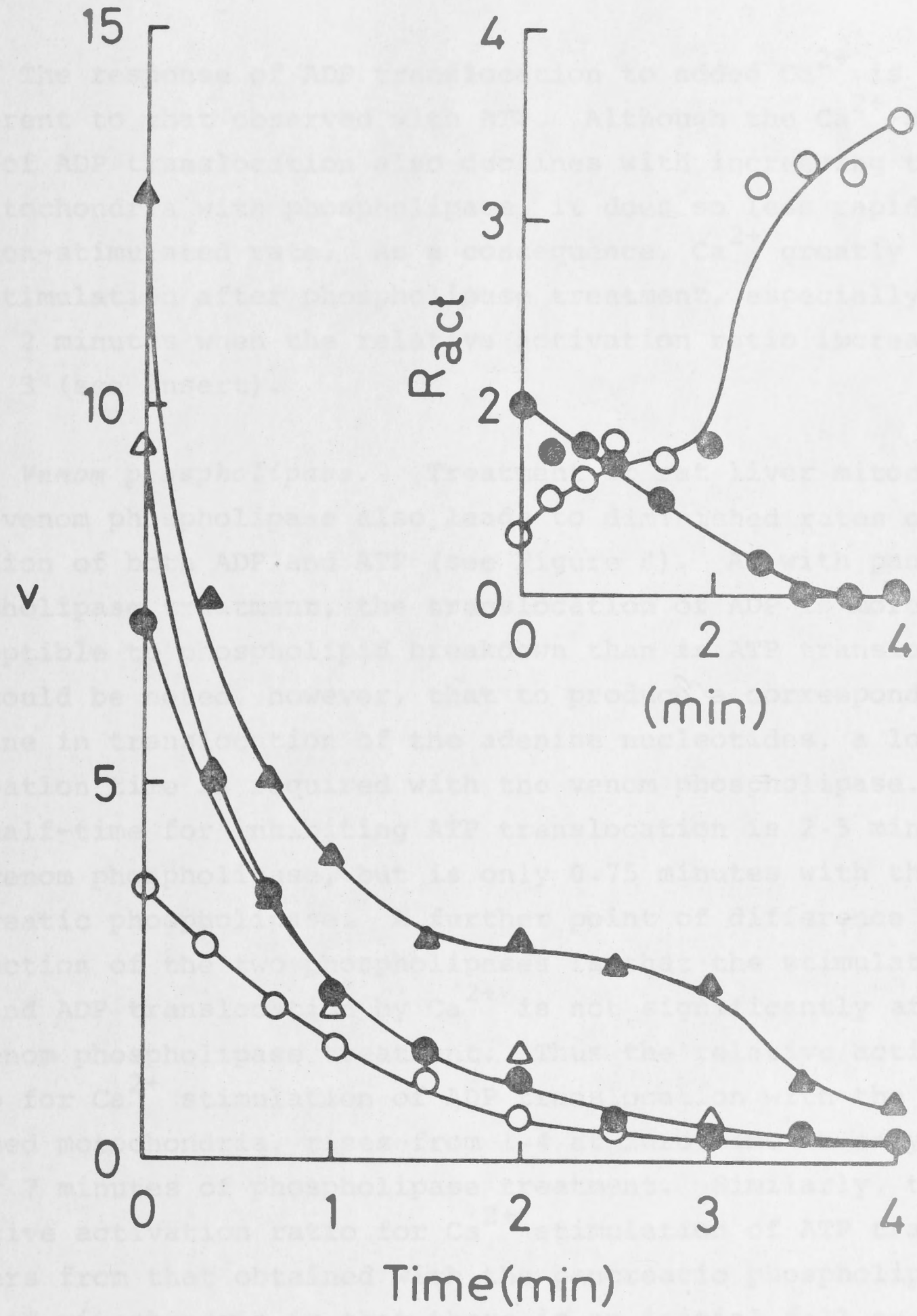

Figure 7. *Effect of pancreatic phospholipase treatment on the activity of the adenine nucleotide translocase.*

Mitochondria were treated with phospholipase as described in Section B for the times indicated and translocase activity determined in the absence (open symbols) or presence (closed symbols) of 200μM Ca^{2+}. △ ▲ , 200μM ADP; ○ ● , 200μM ATP.

Data in the insert show the relative activation ratio after the indicated times of phospholipase treatment. ● , ATP system; ○ , ADP system. V refers to the rate of adenine nucleotide translocation in nmoles/min per mg protein.

The response of ADP translocation to added Ca^{2+} is quite different to that observed with ATP. Although the Ca^{2+}-stimulated rate of ADP translocation also declines with increasing treatment of mitochondria with phospholipase, it does so less rapidly than the non-stimulated rate. As a consequence, Ca^{2+} greatly enhances ADP stimulation after phospholipase treatment, especially after about 2 minutes when the relative activation ratio imcreases to about 3 (see insert).

Venom phospholipase. Treatment of rat liver mitochondria with venom phospholipase also leads to diminished rates of translocation of both ADP and ATP (see Figure 8). As with pancreatic phospholipase treatment, the translocation of ADP is more susceptible to phospholipid breakdown than is ATP translocation. It should be noted, however, that to produce a corresponding decline in translocation of the adenine nucleotides, a longer incubation time is required with the venom phospholipase. Thus the half-time for inhibiting ATP translocation is 2·5 minutes with the venom phospholipase, but is only 0.75 minutes with the pancreatic phospholipase. A further point of difference between the action of the two phospholipases is that the stimulation of ATP and ADP translocation by Ca^{2+} is not significantly affected by venom phospholipase treatment. Thus the relative activation ratio for Ca^{2+} stimulation of ADP translocation with the venom treated motochondria, rises from 1·4 at zero time to only 1·7 after 7 minutes of phospholipase treatment. Similarly, the relative activation ratio for Ca^{2+} stimulation of ATP translocation differs from that obtained with the pancreatic phospholipase-treated mitochondria in that there is an initial fall to 1·5 at 1 minute and thereafter a gradual rise to the original level after 5 minutes of incubation.

Kinetic parameters for Ca^{2+}-stimulated adenine nucleotide translocation after different times of phospholipase treatment of rat liver mitochondria

Quantitative information on the degree to which Ca^{2+} stimulates adenine nucleotide translocation together with that on the affinity of the stimulation for Ca^{2+} (see Section C) was obtained after various times of treatment of the mitochondria, with pancreatic and with venom phsopholipase. To obtain this information ATP and ADP translocation was measured in the presence of

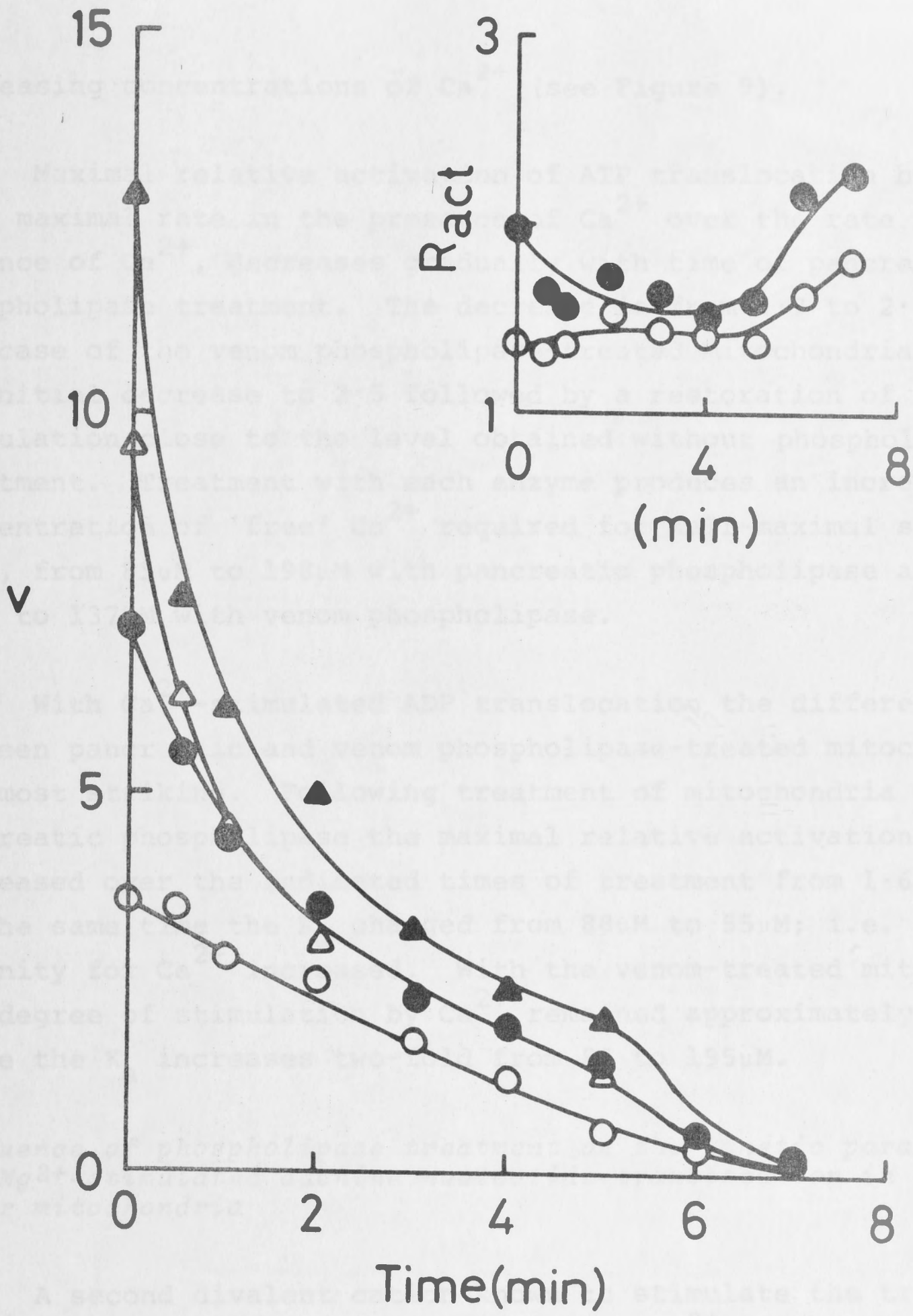

Figure 8. *Effect of venom phospholipase treatment on the activity of the adenine nucleotide translocase*

The entire procedure was carried out as described in the legend to Figure 7 and in Section B except that venom phospholipase was used. Translocation of 200μM ATP (circles) or ADP triangles was determined in the absence (open symbols) or presence (closed symbols) of 200μM Ca^{2+}. Inset data is as described in the legend to Figure 7. V refers to the rate of adenine nucleotide translocation in nmoles/min per mg protein.

increasing concentrations of Ca^{2+} (see Figure 9).

Maximal relative activation of ATP translocation by Ca^{2+}, i.e. maximal rate in the presence of Ca^{2+} over the rate in the absence of Ca^{2+}, decreases gradually with time of pancreatic phospholipase treatment. The decrease is from 4·7 to 2·8. In the case of the venom phospholipase-treated mitochondria there is an initial decrease to 2·5 followed by a restoration of the stimulation close to the level obtained without phospholipase treatment. Treatment with each enzyme produces an increase in the concentration of 'free' Ca^{2+} required for half-maximal stimulation (K_a), from 85μM to 198μM with pancreatic phospholipase and from 85μM to 137μM with venom phospholipase.

With Ca^{2+}-stimulated ADP translocation the differences between pancreatic and venom phospholipase-treated mitochondria are most striking. Following treatment of mitochondria with pancreatic phospholipase the maximal relative activation by Ca^{2+} increased over the indicated times of treatment from 1·6 to 3·0. At the same time the K_a changed from 88μM to 55μM; i.e. the affinity for Ca^{2+} increased. With the venom-treated mitochondria the degree of stimulation by Ca^{2+} remained approximately the same while the K_a increases two-fold from 88 to 195μM.

Influence of phospholipase treatment on the kinetic parameters for Mg^{2+}-stimulated adenine nucleotide translocation in rat liver mitochondria

A second divalent cation known to stimulate the translocation of both ATP and ADP into mitochondria is Mg^{2+}. Stimulation of ADP translocation by Mg^{2+}, however, is greater than that of ATP translocation (cf. Ca^{2+}) and requires much higher metal concentrations (see ref. 165 and Section C). This is shown by kinetic constants obtained for the control as indicated in Figure 10.

ATP translocation

Pancreatic phospholipase treatment of rat liver mitochondria has little effect on the maximal relative activation by Mg^{2+} of ATP translocation (Figure 10a). However, the affinity for Mg^{2+} decreases with increasing incubation time; the K_a for 'free' Mg^{2+} increases from 0·5 to 1·82 mM. Following venom phospholipase

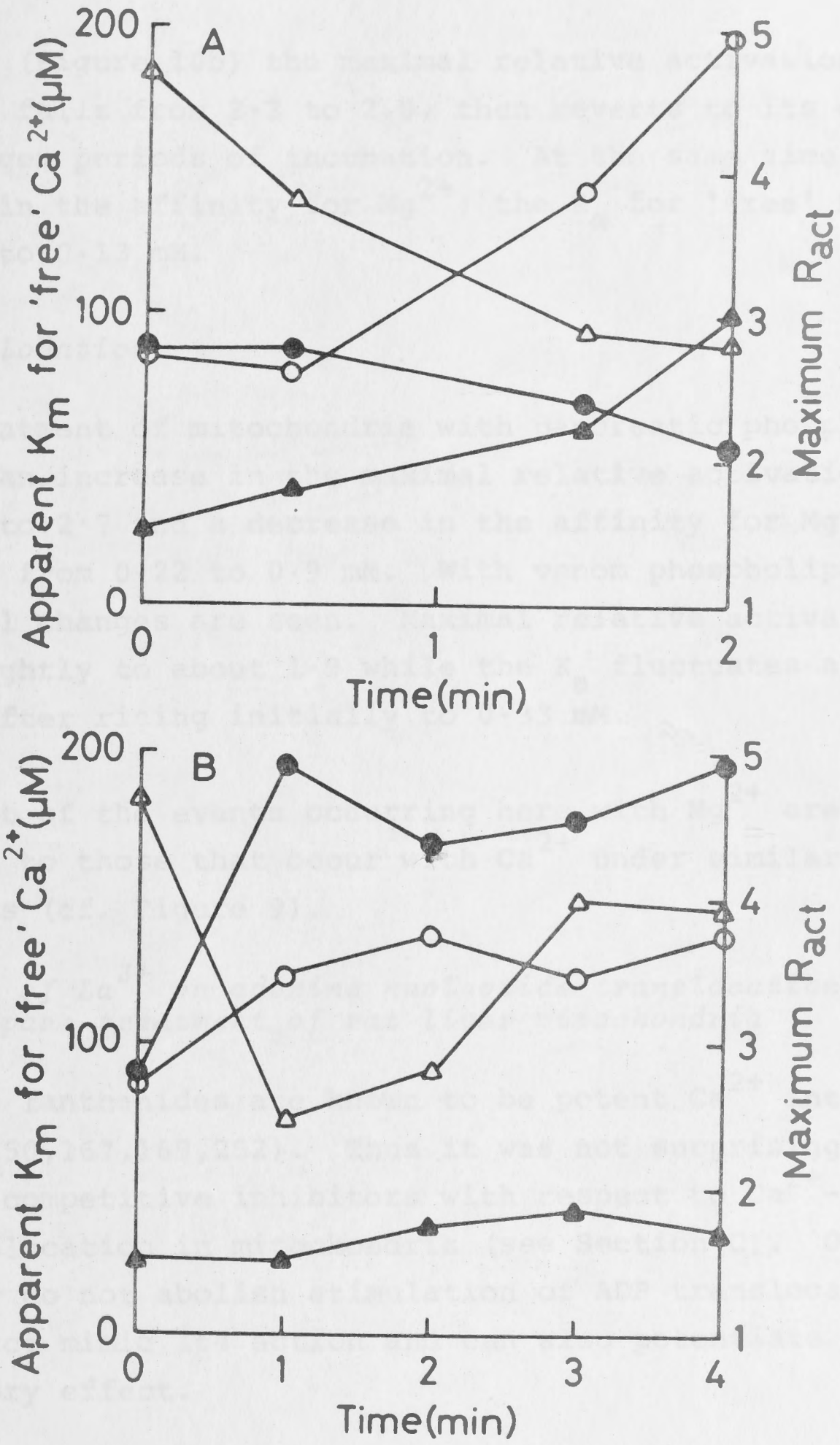

Figure 9. *Effect of phospholipase treatment on the kinetic constants for Ca^{2+}-stimulated adenine nucleotide translocation*

The experiments were carried out essentially as described in the legend to Figure 7 except that the translocation of 200μM ADP (closed symbols) and 200μM ATP (open symbols) was determined in the presence of a range of Ca^{2+} concentrations from 0-600μM. The kinetic parameters were determined from double reciprocal plots of the data obtained after the indicated times of phospholipase treatment. Maximal relative activation (triangles) is expressed as the maximal rate of translocation in the presence of Ca^{2+} over the rate of translocation in the absence of Ca^{2+}. K_a (circles) represents the apparent K_m values for Ca^{2+}. All values are for 'free' Ca^{2+}. A and B represent mitochondria treated with pancreatic and venom phospholipase, respectively.

treatment (Figure 10b) the maximal relative activation of Mg^{2+} initially falls from 2·3 to 2·0, then reverts to its original value after longer periods of incubation. At the same time there is an increase in the affinity for Mg^{2+}; the K_a for 'free' Mg^{2+} decreases from 0·5 to 0·13 mM.

ADP translocation

Treatment of mitochondria with pancreatic phospholipase produces an increase in the maximal relative activation by Mg^{2+} from 2·1 to 2·7 and a decrease in the affinity for Mg^{2+}; the K_a increases from 0·22 to 0·9 mM. With venom phospholipase treatment only small changes are seen. Maximal relative activation by Mg^{2+} falls slightly to about 1·9 while the K_a fluctuates a little to 0·15 mM after rising initially to 0·33 mM.

Most of the events occurring here with Mg^{2+} are thus quite different to those that occur with Ca^{2+} under similar experimental conditions (cf. Figure 9).

Influence of La^{3+} on adenine nucleotide translocation after phospholipase treatment of rat liver mitochondria

The lanthanides are known to be potent Ca^{2+} antagonists (16,149,150,167,169,252). Thus it was not surprising to find that they are competitive inhibitors with respect to Ca^{2+}-stimulated ATP translocation in mitochondria (see Section C). On the other hand they do not abolish stimulation of ADP translocation by Ca^{2+} but in fact mimic its action and can also potentiate its stimulatory effect.

ATP translocation

Data in Figure 11a,b show how the affinity of La^{3+} for inhibition of Ca^{2+}-stimulated ATP translocation, changes with increasing time of treatment of rat liver mitochondria with the pancreatic and venom phospholipases. As noted earlier (Section C) that portion of ATP translocation which is stimulated by Ca^{2+} has a very high affinity for La^{3+} (K_i approximately 3·5 μM). However, after treatment of rat liver mitochondria with the phospholipases there is an overall decrease in the affinity for La^{3+}. This is seen especially in the case of pancreatic phospholipase treated

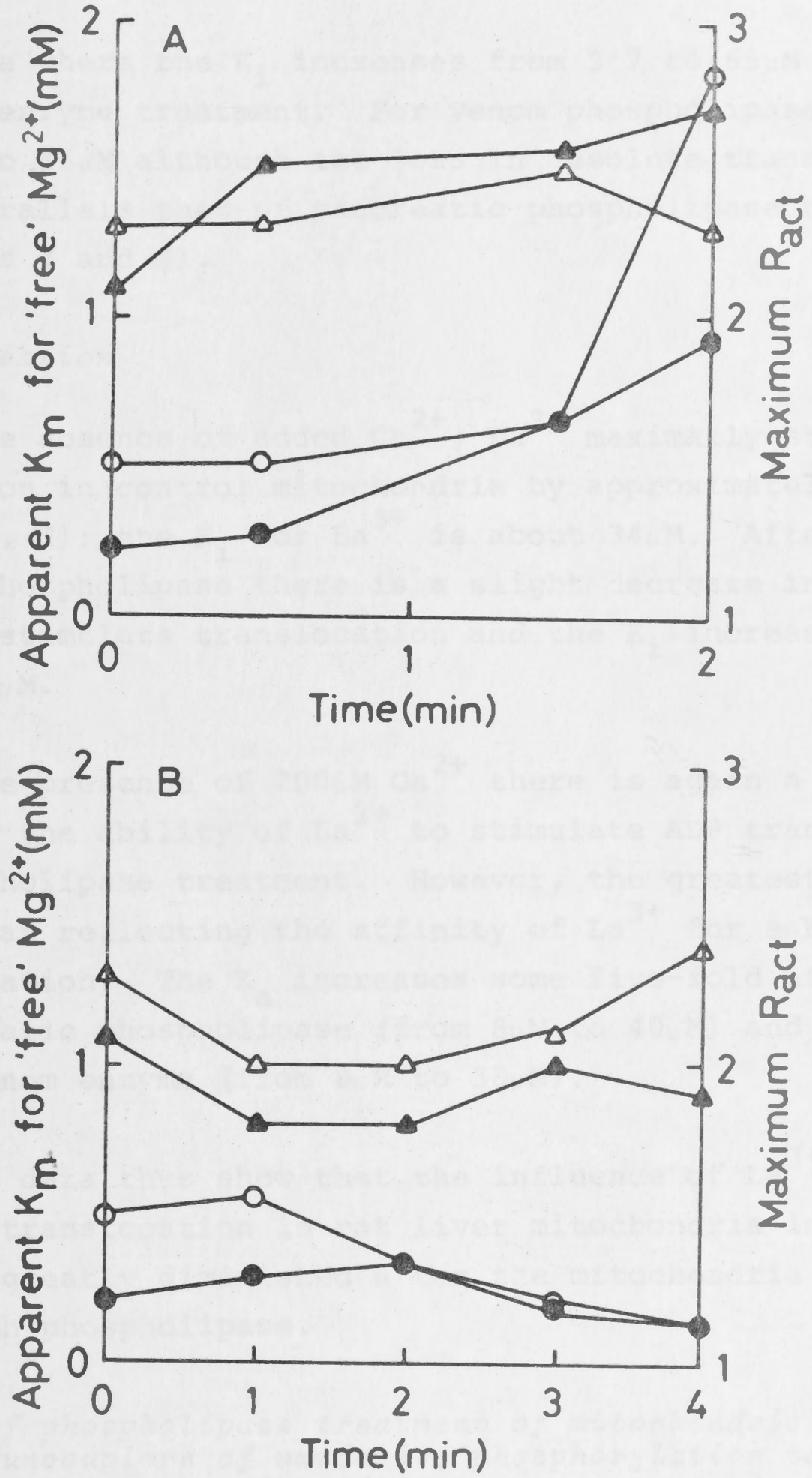

Figure 10. *Effect of phospholipase treatment on the kinetic constants for Mg^{2+}-stimulated adenine nucleotide translocation*

The experiments were carried out as described in the legend to Figure 7 except that the translocation of 200μM ADP (closed symbols) and 200μM ATP (open symbols) was determined in the presence of a range of Mg^{2+} concentrations from 0-8 mM. The kinetic parameters were determined from double-reciprocal plots of the data obtained after the indicated times of phospholipase treatment. Maximal relative activation (triangles) is expressed as in Figure 9. K_a (circles) represents the apparent K_m values for Mg^{2+}. All values are for 'free' Mg^{2+}. A and B represent mitochondria treated with pancreatic and venom phospholipase, respectively.

mitochondria where the K_i increases from 3·7 to 65μM after 1·5 minutes of enzyme treatment. For venom phospholipase the K_i increases to 35μM although the loss in absolute translocase activity parallels that of pancreatic phospholipase treatment (see Figures 7 and 8).

ADP translocation

In the absence of added Ca^{2+}, La^{3+} maximally stimulates ADP translocation in control mitochondria by approximately 50% (Figure 11c, d); the K_i for La^{3+} is about 34μM. After treatment with each phospholipase there is a slight decrease in the ability of La^{3+} to stimulate translocation and the K_i increases slightly to about 40μM.

In the presence of 200μM Ca^{2+} there is again a slight decrease in the ability of La^{3+} to stimulate ADP translocation after phospholipase treatment. However, the greatest change to occur is that reflecting the affinity of La^{3+} for enhancing the Ca^{2+} stimulation. The K_a increases some five-fold after treatment with pancreatic phospholipase (from 8μM to 40μM) and some four-fold with the venom enzyme (from 8μM to 30μM).

These data thus show that the influence of La^{3+} on adenine nucleotide translocation in rat liver mitochondria in the presence of Ca^{2+} is greatly diminished after the mitochondria have been treated with phospholipase.

Influence of phospholipase treatment of mitochondria on the ability of uncouplers of oxidative phosphorylation to stimulate adenine nucleotide translocation

Uncouplers of oxidative phosphorylation such as CCCP stimulate ATP but not ADP translocation in mammalian mitochondria (199,239). Data in Figure 12 show the influence of CCCP on ATP and ADP translocation following different times of venom and pancreatic phospholipase treatment of the mitochondria. The large stimulation of ATP translocation by CCCP seen in the control mitochondria is diminished after treatment of these organelles with pancreatic phospholipase (see insert to Figure 12a). With ADP by contrast, there is an increase in the relative activation ratio from 0·75 to 1·5 after about 1 minute of phospholipase treatment.

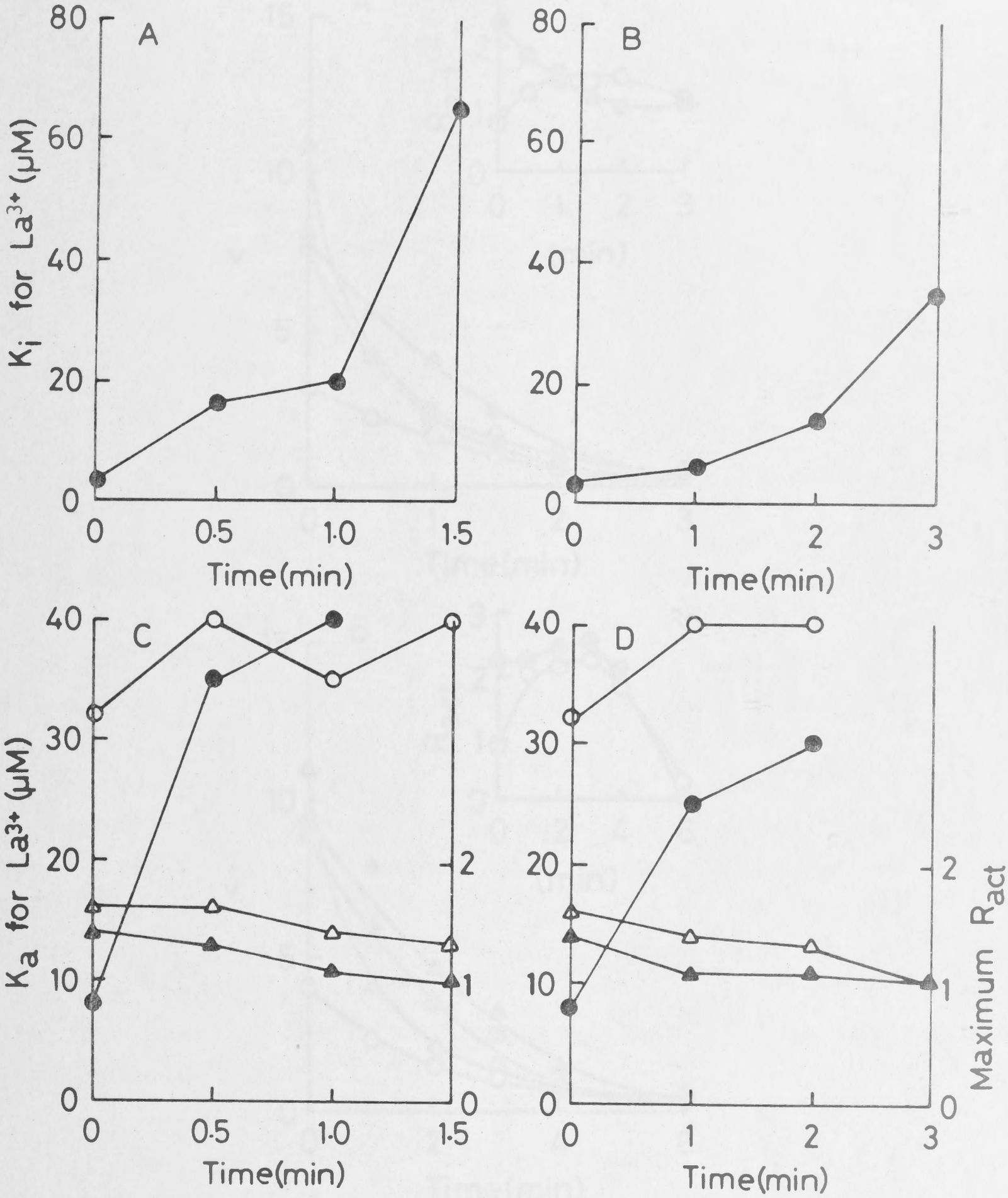

Figure 11. *Effect of phospholipase treatment on the ability of La^{3+} to inhibit Ca^{2+}-stimulated ATP translocation and promote Ca^{2+}-stimulation of ADP translocation.*

The experimental system was as described in Figure 7 except that in the first set of experiments the translocation of 200μM ATP stimulated by 200μMCa^{2+} was examined in the presence of varying amounts of La^{3+} after the indicated times of treatment of mitochondria with pancreatic (A) and venom (B) phospholipase. In the second set of experiments mitochondria were treated with pancreatic (C) and venom (D) phospholipase and translocation of 200μM ADP in the absence (open symbols) or presence (closed symbols) of 200μM Ca^{2+} measured in the presence of varying concentrations of La^{3+}. Maximal relative activation is denoted by triangles and K_a for La^{3+} denoted by circles. The constants were obtained from double-reciprocal plots of the original data.(see Fig.11,Section C).

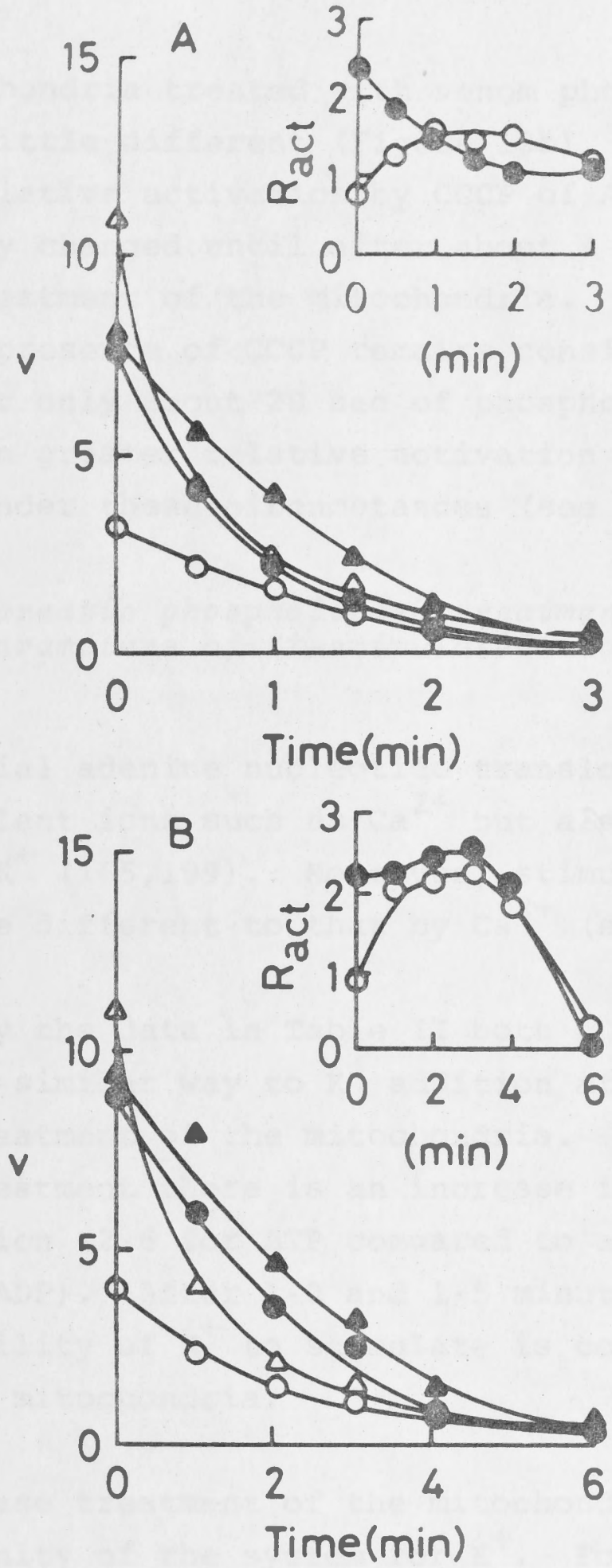

Figure 12. *Effect of phospholipase treatment on ability of CCCP to influence adenine nucleotide translocation.*

The experimental conditions were essentially those described in the legend to Figures 7 and 8 except that CCCP (5μM) was present (closed symbols) as indicated. Mitochondria were treated with pancreatic (A) or venom (B) phospholipase. Circles and triangles represent translocation of 200μM ATP and 200μM ADP respectively. Data in the insets to each of the Figures represent the relative activation ratio; i.e. rate of translocation in the presence of CCCP over the rate of translocation in the absence of CCCP at the different times of phospholipase treatment. ● , ATP system; ○ , ADP system. V refers to the rate of adenine nucleotide translocation in nmoles/min per mg protein.

With mitochondria treated with venom phospholipase the situation is a little different (Figure 12b). In the first instance, the relative activation by CCCP of ATP translocation is not significantly changed until after about 4 minutes or longer of phospholipase treatment of the mitochondria. Secondly, ATP translocation in the presence of CCCP remains considerably greater than that of ADP after only about 20 sec of phospholipase treatment. Moreover, an even greater relative activation of ADP translocation by CCCP occurs under these circumstances (see insert to Figure 12b).

Influence of pancreatic phospholipase treatment of mitochondria on the kinetic parameters of K^+-stimulated adenine nucleotide translocation

Mitochondrial adenine nucleotide translocation is stimulated not only by bivalent ions such as Ca^{2+} but also by monovalent cations such as K^+ (165,199). Moreover, stimulation by K^+ involves a mechanism quite different to that by Ca^{2+} (see Section C).

As shown by the data in Table II both ADP and ATP translocation respond in a similar way to K^+ addition after pancreatic phospholipase treatment of the mitochondria. After 0·5 minutes of phospholipase treatment there is an increase in the maximal relative activation (2·6 for ATP compared to a control value of 1·8 and 1·6 for ADP). After 1·0 and 1·5 minutes of phospholipase treatment the ability of K^+ to stimulate is considerably less than in the untreated mitochondria.

Phospholipase treatment of the mitochondria also results in a decreased affinity of the system for K^+. For ATP translocation the K_a increased from 3·2 to 7mM and for ADP translocation, from 2·6 to 6mM. Essentially similar data were obtained when the mitochondrial phospholipids were hydrolysed using venom phospholipase (data not presented).

Influence of phospholipase treatment on the substrate kinetics of adenine nucleotides

Studies on translocase activity as a function of ADP and ATP concentration indicate that both double reciprocal and Eadie plots give a non-linear relationship indicative of two K_m values for this process, one of high and the other of low affinity (see Section C).

TABLE II

Effect of pancreatic phospholipase treatment on the kinetic parameters for K^+-stimulated translocation

Experiments were performed and results analysed as in the legend to Figure 9. KCl was varied over the concentration range 0-50mM and the medium brought to 200mosmol with appropriate amounts of sucrose. The 'activation ratio' is defined as the maximal translocase activity in the presence of K^+ over that in the absence of K^+.

Adenine nucleotide translocated	Parameter measured	Min. of incubation with phospholipase			
		0	0.5	1.0	1.5
ATP	Activation Ratio	1.8	2.6	1.4	1.0
	K_a (mM)	3.2	4.0	7.0	-
ADP	Activation Ratio	1.6	1.8	1.2	1.0
	K_a (mM)	2.6	5.0	6.0	

Data presented in Figure 13 show the effect of phospholipase treatment on the kinetic parameters of the translocase with respect to the substrates, ADP and ATP. As expected from previous results the V_{max} values decreased with increasing times of incubation with the respective phospholipases. This decrease is more pronounced for ADP. With both enzymes the K_m for the high affinity portion of the translocation process increased with phospholipase treatment; e.g. from 20 to 53μM and 13 to 26μM for ATP and ADP translocation respectively after 1·5 minutes of pancreatic enzyme treatment. The value for the high K_m, or low affinity portion of exchange, did not change appreciably.

Influence of oleic acid and lysophospholipids on adenine nucleotide translocation in rat liver mitochondria

In control experiments the effect of the products of phospholipase A hydrolysis, i.e. fatty acid and lysophospholipids, on the translocation of ATP and ADP was examined.

Data in Figure 14a, b, shows the effect of the products of phospholipase digestion lysophosphatidylethanolamine and lysophosphatidylcholine on the translocation of ATP and ADP. Lysophosphatidylcholine is more effective than lysophosphatidyl-ethanolamine in inhibiting the rate of translocation; in the case of ATP, inhibition is 65% with the former and 35% with the latter at a concentration of 80μM. This difference is even more pro-nounced with ADP translocation which is not affected by lysophosphatidylethanolamine whilst phosphatidylcholine inhibits approximately 57% at an equivalent concentration to that mentioned above. The addition of Ca^{2+} to the ATP system results in a decreased rate of inhibition of ATP translocation; lysophosphatidylethanolamine inhibits some 35% in the absence of Ca^{2+} and only 8% in its presence.

The other product of phospholipase A digestion of phospholipids is fatty acid. As shown in Figure 15 oleic acid inhibits the translocation of both ADP and ATP. Data in Table III summarises the kinetic parameters of this inhibition. Maximal % inhibitions varied from 45% in the case of ATP translocation to 67% in the case of ADP. In the presence of Ca^{2+} the maximal % inhibition for both ATP and ADP are approximately the same at 60-61%. These degrees of inhibition are much less than that

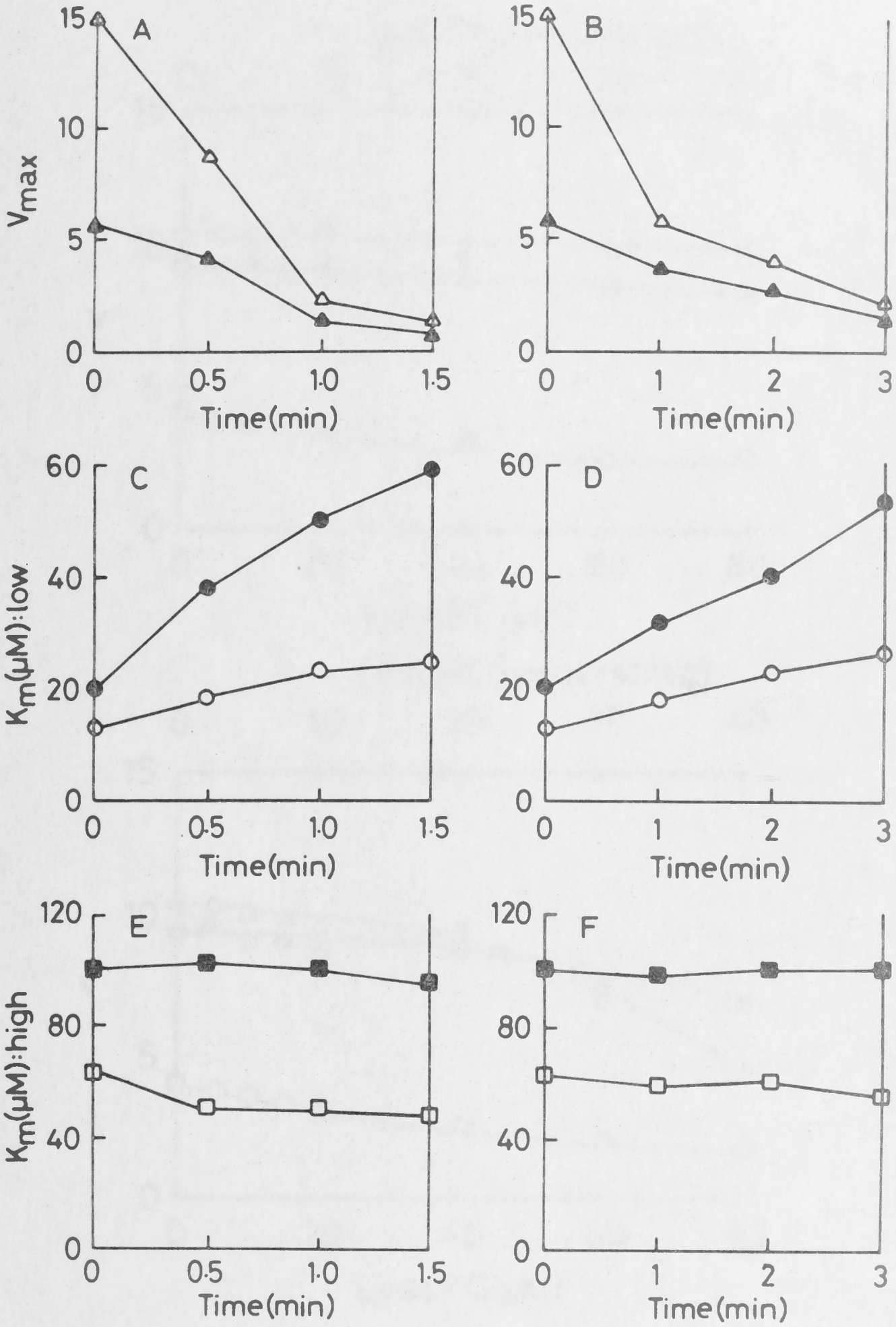

Figure 13. *Effect of phospholipase treatment on the substrate kinetics of adenine nucleotide translocation.*

Mitochondria were treated with pancreatic (A,C,E) or venom (B,D,F) phospholipase and translocation of varying concentrations (2-600μM) of ADP (open symbols) and ATP (closed symbols) measured. The kinetic constants were calculated from double-reciprocal plots of the data. Values in Figures A and B represent maximal velocities of ADP and ATP translocation; those in Figures C and D high affinity K_m and those in Figures E and F low affinity K_m for ADP and ATP translocation.

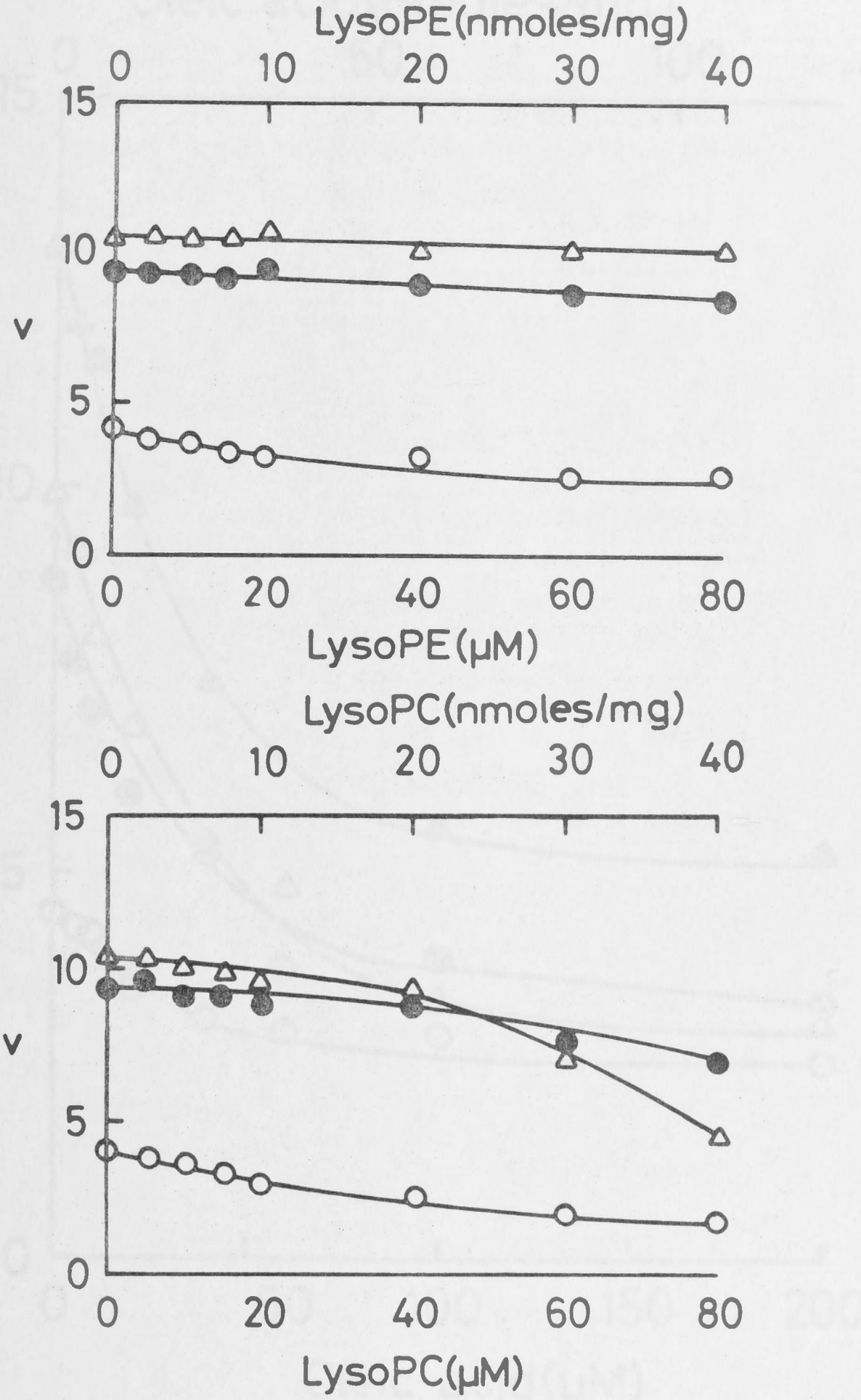

Figure 14. *Effect of lysophosphatidylcholine and lysophosphatidylethanolamine on the translocation of adenine nucleotides.*

Mitochondria were tested for their ability to translocate adenine nucleotides as in Section B at a temperature of 4°C. Concentrations of lyso compounds as indicated were preincubated with the mitochondria for 2 minutes before the initiation of the experiments. A, lysophosphatidylethanolamine; B, lysophosphatidylcholine. ○ 200μM ATP, △ 200μM ADP, ● 200μM ATP + 200μM Ca^{2+}. V refers to the rate of adenine nucleotide translocation in nmoles/min per mg protein.

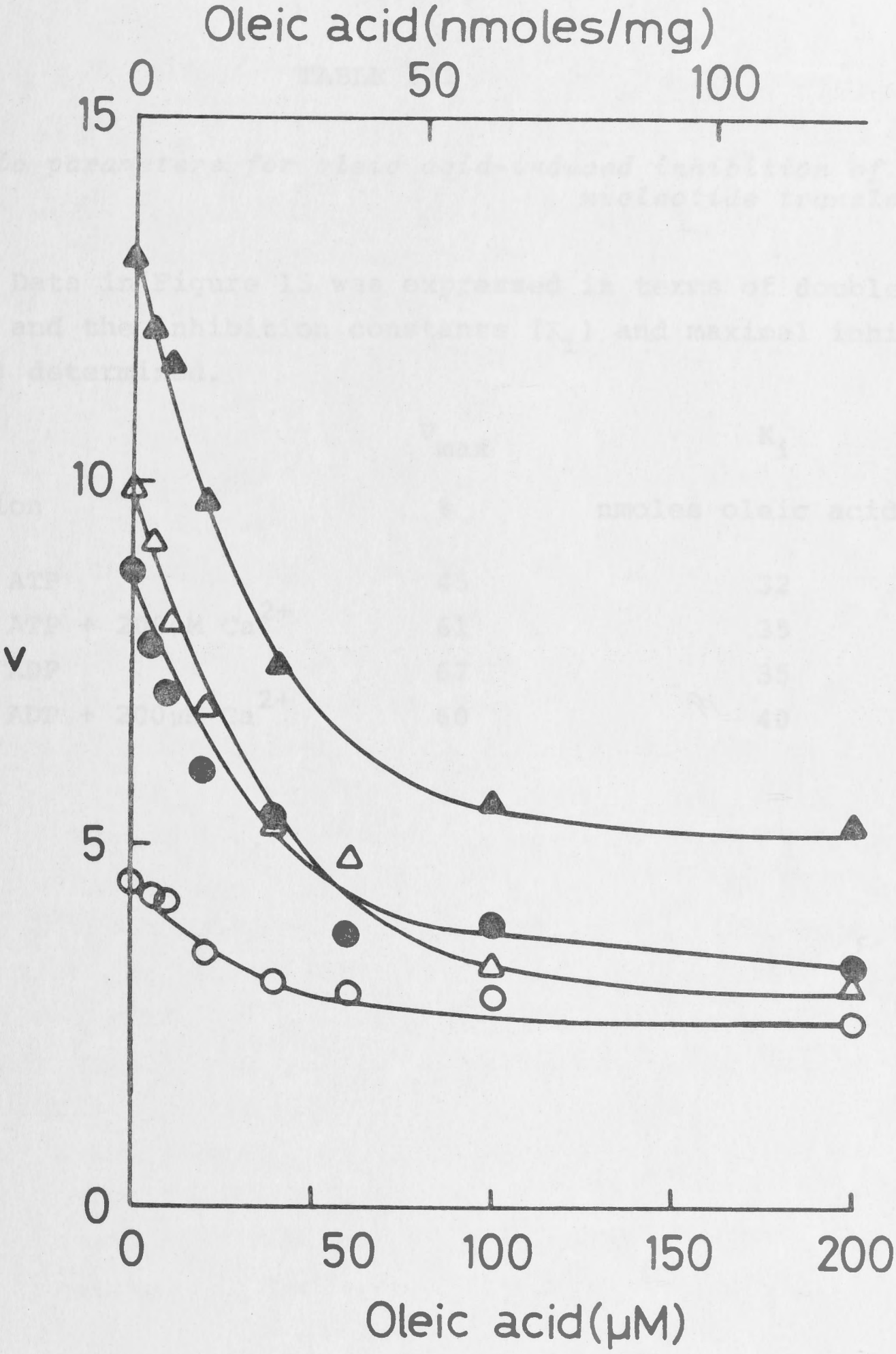

Figure 15. *Effect of oleic acid on the translocation of adenine nucleotides.*

Mitochondria were treated as described in the legend to Figure 14 except that oleic acid was added and its concentration varied. ○ 200μM ATP, △ 200μM ADP, ● 200μM ATP + 200μM Ca^{2+}, ▲ 200μM ADP + 200μM Ca^{2+}. V refers to the rate of adenine nucleotide translocation in nmoles/min per mg protein.

TABLE III

Kinetic parameters for oleic acid-induced inhibition of adenine nucleotide translocation

Data in Figure 15 was expressed in terms of double-reciprocal plots and the inhibition constants (K_i) and maximal inhibition (V_{max}) determined.

	V_{max}	K_i
Addition	%	nmoles oleic acid/mg protein
200μM ATP	45	32
200μM ATP + 200μM Ca^{2+}	61	35
200μM ADP	67	35
200μM ADP + 200μM Ca^{2+}	60	40

reported by Wojtczak *et al* (273,274) who found that the addition of 25-50 nmoles of oleate per mg of protein resulted in an 80-90% inhibition of ATP translocation. Half-maximal inhibition as shown in Figure 15 required between 32 to 40 nmoles oleic acid per mg of protein. These values compare favourably with that concentration found by Wojtczak *et al* to half-maximally inhibit mitochondrial ATPase activity, an indirect measure of the rate of translocation (see ref. 94).

Figure 16 shows the effect of treatment of mitochondria with a single concentration of fatty acid or lysophospholipid on the translocation of ATP and ADP. This experiment differed from those described in Figures 14 and 15 in that the effect of a subsequent BSA wash on the translocation rates was investigated. In order to perform this type of experiment the mitochondria were first subjected to their respective treatments and then separated from the medium by centrifugation and resuspended as described in the legend to Figure 16. The concentration of the effectors used was approximately the same as would be expected to be formed after the action of venom phospholipase for 6 minutes. The following points emerge from the control data, i.e. without BSA wash, presented in Figure 16. Firstly, treatment of mitochondria with the lyso compounds produces only slight inhibition of adenine nucleotide translocation, compared to oleic acid treatment especially when assayed in the presence of Ca^{2+}. Secondly, the loss of translocase activity measured in the presence of Ca^{2+} or CCCP was not on a *pro rata* basis compared to that loss observed in the absence of effector agents. In the case of ATP translocation oleic acid induced a 68% fall in the rate in the absence of CCCP and 84% in its presence. Similar results were obtained using ADP, and mitochondria that had been treated with lyso compounds. Subsequent treatment of the mitochondrial preparations with BSA after the addition of oleic acid or lyso compounds relieved the inhibition of the translocation process. The translocation of ATP was more resistant to reversal than that of ADP; for example in the absence of effectors and in fatty acid-treated mitochondria the inhibition in the case of the former decreased from 68% to only 65% whilst it went from 83% to 50% with the latter. BSA produced a potentiation of the Ca^{2+}-stimulation with both ATP and ADP, i.e. the reversal of the inhibition was greater when Ca^{2+} was also present in the medium. Another important point was that ADP translocation was

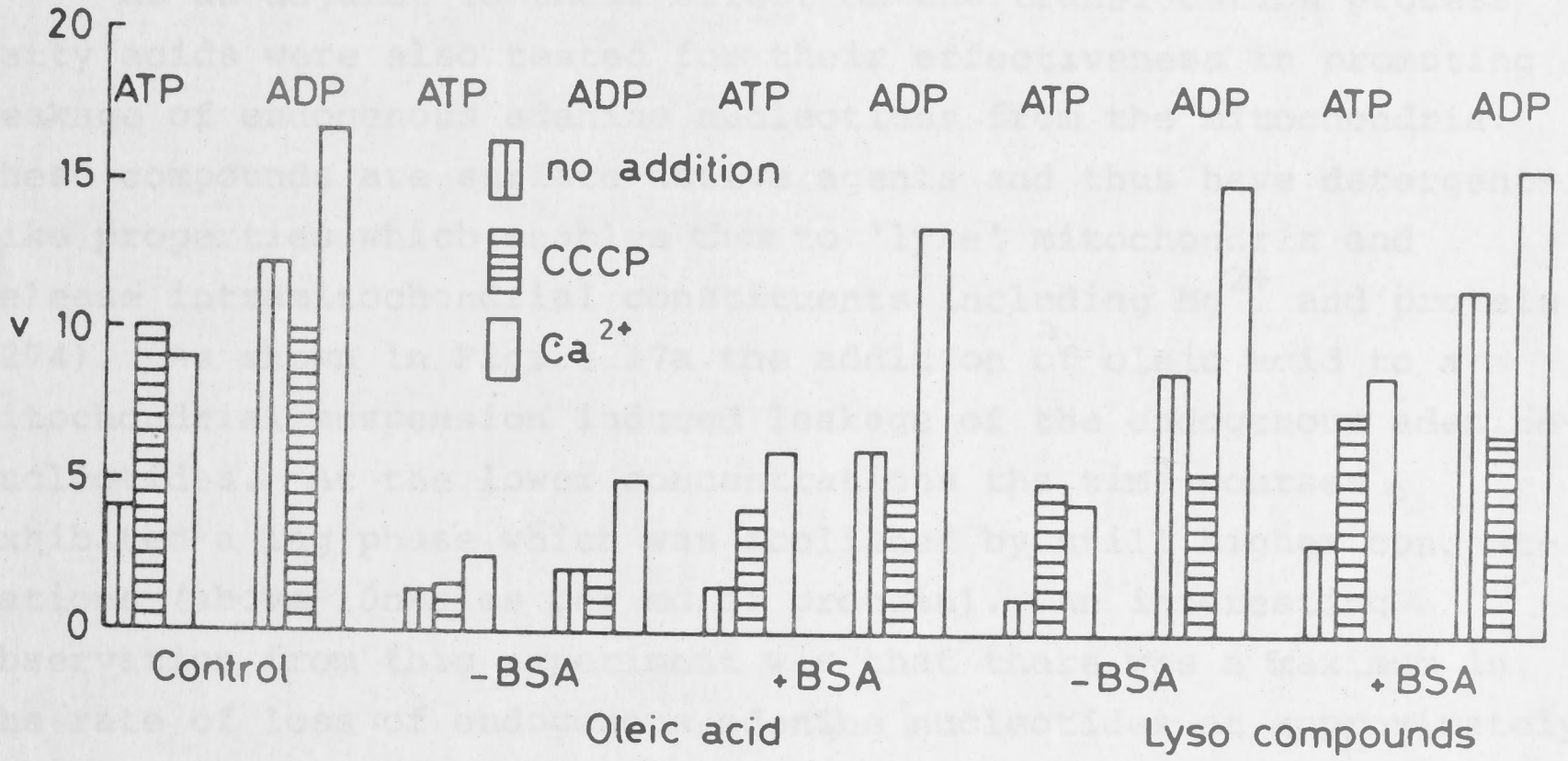

Figure 16. *Adenine nucleotide translocation in mitochondria pre-treated with fatty acids or with lysophospholipids.*

Rat liver mitochondria (10 mg protein/ml) were incubated with oleic acid (0.5 mM) or with a mixture of lysophosphatidyl ethanolamine (0.25 mM) and lysophosphatidyl choline (0.25 mM) at 25°C. After 2 minutes the mitochondria were diluted 10-fold with a wash medium containing 200 mM sucrose, 2 mM Hepes-tris (pH 7.4), 0.5 mM EGTA plus or minus 0.5% BSA and kept on ice for 5 minutes. The mitochondria were packed by centrifugation, resuspended and adenine nucleotide translocation measured in the presence of Ca^{2+} (200μM) or CCCP (5μM) and 200μM ATP or ADP as indicated. V refers to the rate of adenine nucleotide translocation in nmoles/min per mg protein.

not stimulated by CCCP after either fatty acid or lyso treatment, whereas ATP translocation was stimulated significantly by this uncoupler under these conditions. All of these events then are clearly different to those which occur after phospholipase A treatment of the rat liver mitochondria.

Influence of phospholipase A hydrolysis products on mitochondrial integrity

As an adjunct to their effect on the translocation process fatty acids were also tested for their effectiveness in promoting leakage of endogenous adenine nucleotides from the mitochondria. These compounds are surface-active agents and thus have detergent-like properties which enables them to 'lyse' mitochondria and release intramitochondrial constituents including Mg^{2+} and protein (274). As shown in Figure 17a the addition of oleic acid to a mitochondrial suspension induced leakage of the endogenous adenine nucleotides. At the lower concentrations the time-courses exhibited a lag phase which was abolished by still higher concentrations (above 10nmoles per mg of protein). An interesting observation from this experiment was that there was a maximum in the rate of loss of endogenous adenine nucleotides at approximately 30 nmoles of oleic acid per mg of protein as shown in Figure 17b. This value is not significantly different from those values obtained for the half-maximal inhibition of adenine nucleotide translocation (see Table III). The concentration of oleic acid needed to half-maximally stimulate the leakage was at least 25 nmoles per mg of protein. Another important observation was that the response to increasing fatty acid was sigmoidal; the mitochondrial membranes may bind a significant amount of oleic acid without it having any detrimental effects on their permeability. Similar results to those described above were also found using linoleic and arachidonic acids. In contrast to fatty acids, lyso compounds had virtually no effect on the leakage of adenine nucleotides; after 20 minutes the extent of leakage with 50 nmoles of lysophosphatidylcholine was approximately 10% as compared to 5% in the control.

A maximum in the rate of swelling was also observed on the addition of fatty acid as shown in Figure 18. In this experiment the range of fatty acid concentrations used was higher than that

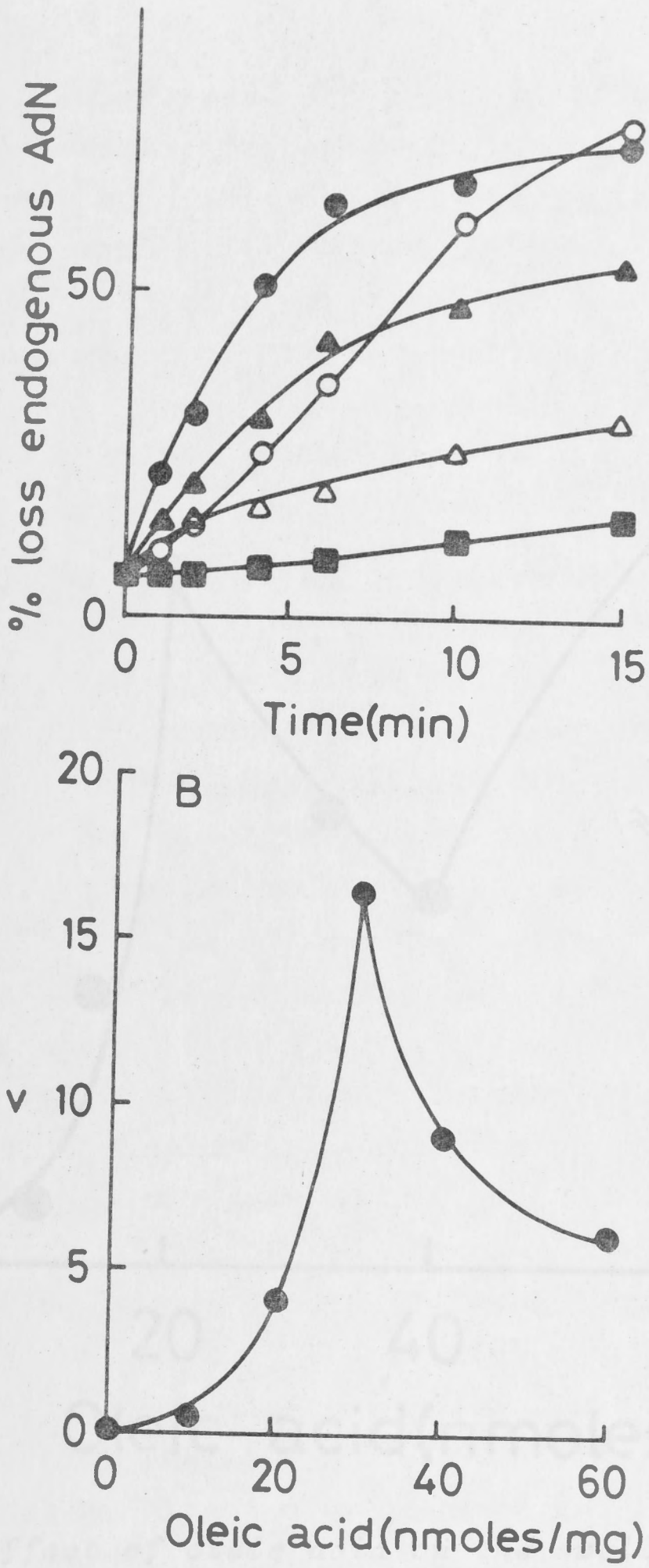

Figure 17. *Effect of oleic acid on the leakage of endogenous adenine nucleotides.*

Mitochondria were incubated in a total volume of 2.0ml as in the legend to Figure 5 in the absence of BSA and with the indicated concentrations of oleic acid. At the times indicated 0.1ml of the suspension was removed and centrifuged in an Eppendorf microfuge for 2.5 minutes. Portions (50µl) of the supernatant solution were counted to determine the release of radioactivity from the mitochondria. A, time-course of release. ■ 10nmoles oleic acid/mg protein, ○ 20nmoles/mg, ● 30nmoles/mg, ▲ 40nmoles/mg, △ 60nmoles/mg; B, initial rates of release of endogenous adenine nucleotides determined from data in A. AdN = adenine nucleotide. V refers to the initial rate of loss of endogenous adenine nucleotide in %/min.

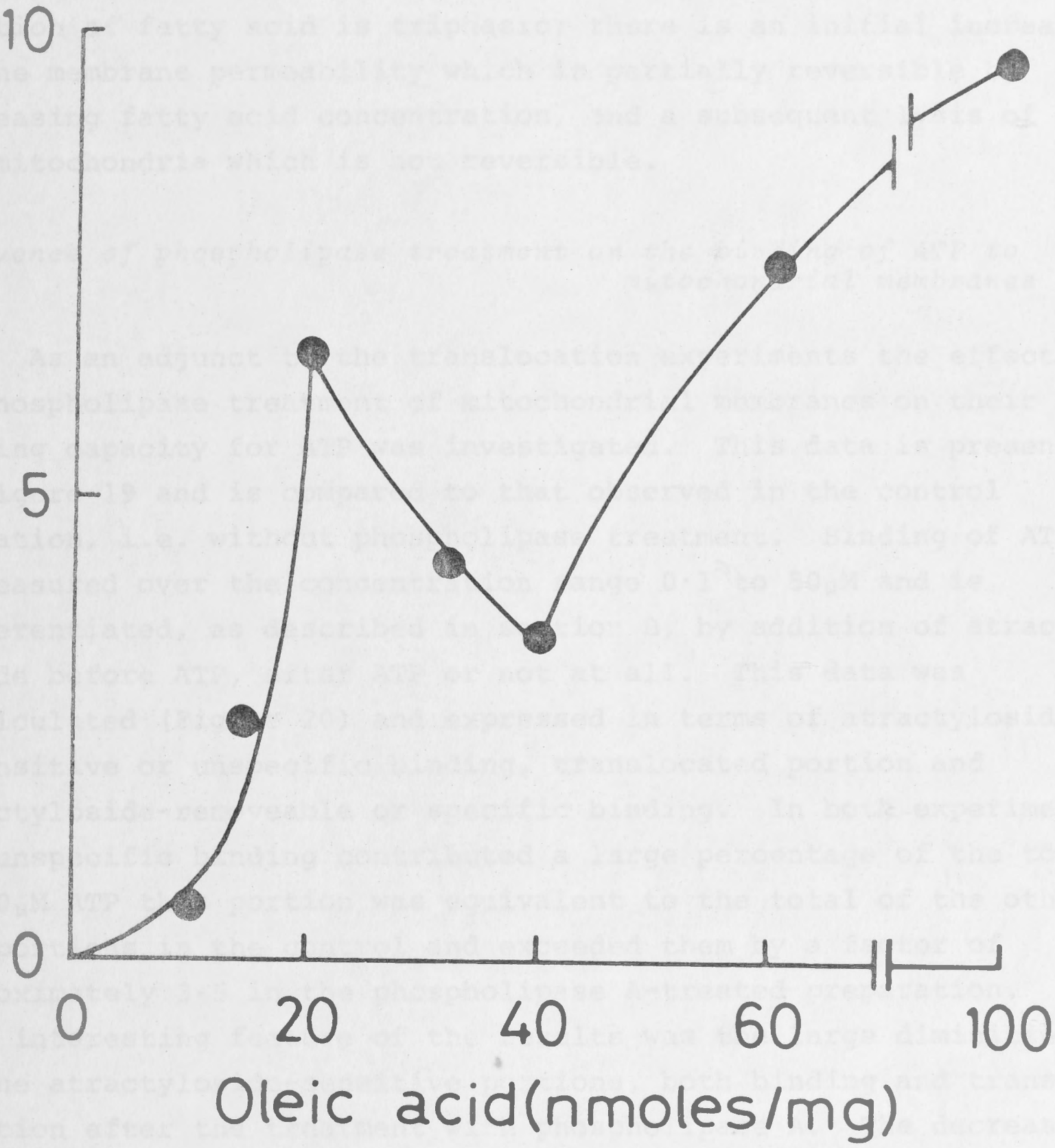

Figure 18. *Effect of oleic acid on the swelling of rat liver mitochondria.*

Mitochondria were incubated as described in the legend to Figure 2 with the indicated amounts of oleic acid and O.D. changes at 520nm recorded. From these recordings the initial rates of swelling were calculated and plotted versus the amount of oleic acid added. V refers to the rate of O.D. decrease in O.D. units X 10^{-1}/min.

in the previous experiment. At the highest concentrations the rate of swelling again increased. Thus the response to the addition of fatty acid is triphasic; there is an initial increase of the membrane permeability which is partially reversible by increasing fatty acid concentration, and a subsequent lysis of the mitochondria which is not reversible.

Influence of phospholipase treatment on the binding of ATP to mitochondrial membranes

As an adjunct to the translocation experiments the effects of phospholipase treatment of mitochondrial membranes on their binding capacity for ATP was investigated. This data is presented in Figure 19 and is compared to that observed in the control situation, i.e. without phospholipase treatment. Binding of ATP is measured over the concentration range 0·1 to 50μM and is differentiated, as described in section B, by addition of atractyloside before ATP, after ATP or not at all. This data was recalculated (Figure 20) and expressed in terms of atractyloside-insensitive or unspecific binding, translocated portion and atractyloside-removeable or specific binding. In both experiments the unspecific binding contributed a large percentage of the total; at 50μM ATP this portion was equivalent to the total of the other two portions in the control and exceeded them by a factor of approximately 3·5 in the phospholipase A-treated preparation. The most interesting feature of the results was the large diminition in the atractyloside-sensitive portions, both binding and translocation after the treatment with phospholipase A. The decrease in the latter is to be expected in view of results presented previously. Binding, in both cases, reaches saturation at approximately 30μM added ATP of 1·2 and 0·24 moles ATP/mole cytochrome a in the control and phospholipase-treated preparations respectively. The size of the atractyloside-insensitive portions does not change significantly since it is mainly due to the occupation of the sucrose space in the pellet. When the binding data contained in Figure 20 is plotted according to the method of Scatchard (216) a non-linear relationship is found indicating two types of binding site with different affinities for ATP. This result is in contrast to that found by Weidemann *et al* (267) who found that beef heart and rat heart mitochondria possessed two types of binding sites whilst rat liver mitochondria only possessed one. More than one type of binding site has also been found by

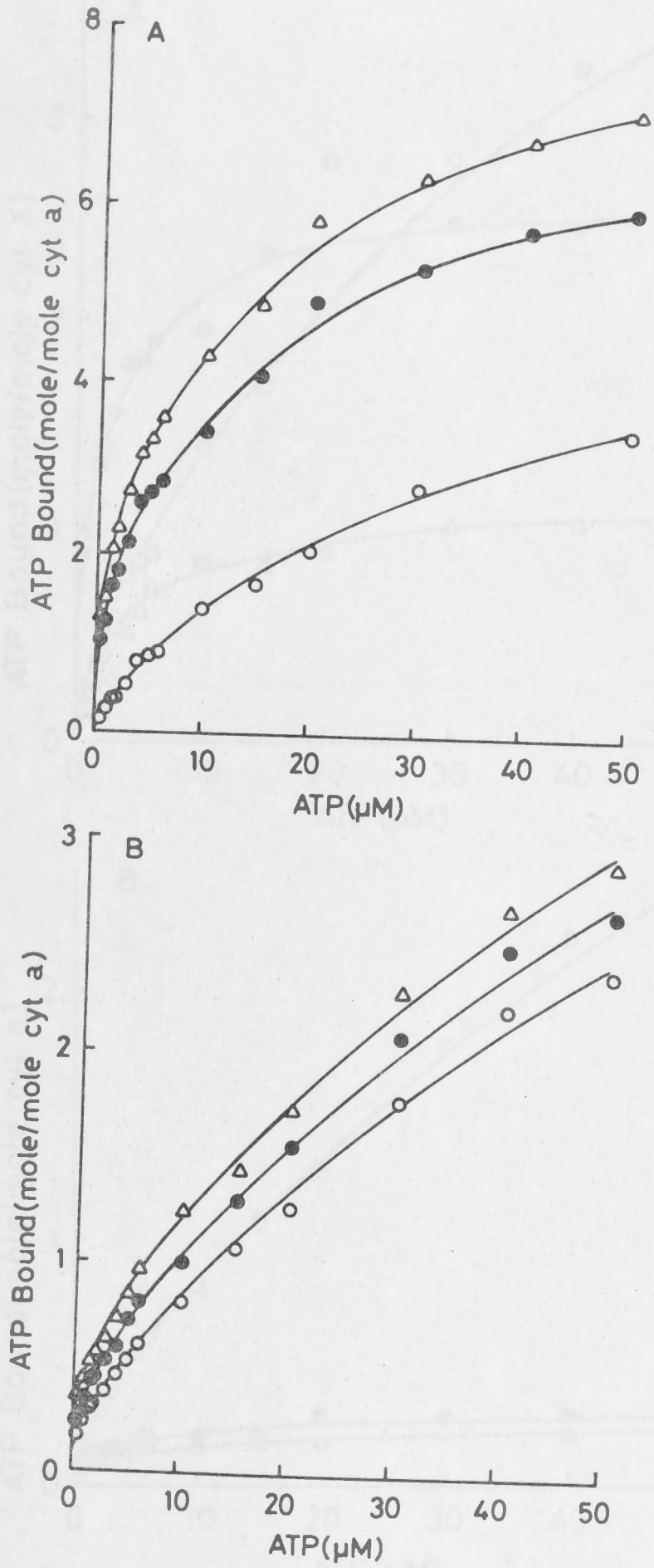

Figure 19. *Effect of phospholipase A treatment on the concentration dependence of [^{3}H]ATP 'uptake' by rat liver mitochondria.*

Mitochondria depleted of their endogenous adenine nucleotides by Pi treatment were incubated as in section B at 0°C with ATP at the concentrations shown. A, control mitochondria; B, Pi-depleted mitochondria were treated with venom phospholipase A (5μg per mg protein) for 15 minutes at 25°C in a medium containing 200mM sucrose, 20mM HEPES, 1% BSA and 2mM Ca^{2+}. At the end of this period they were sedimented, washed once and resuspended ready for binding studies. At each concentration of ATP 50μM atractyloside was either (i) omitted from the medium (△), (ii) added before (○) or (iii) 2 minutes after the ATP (●).

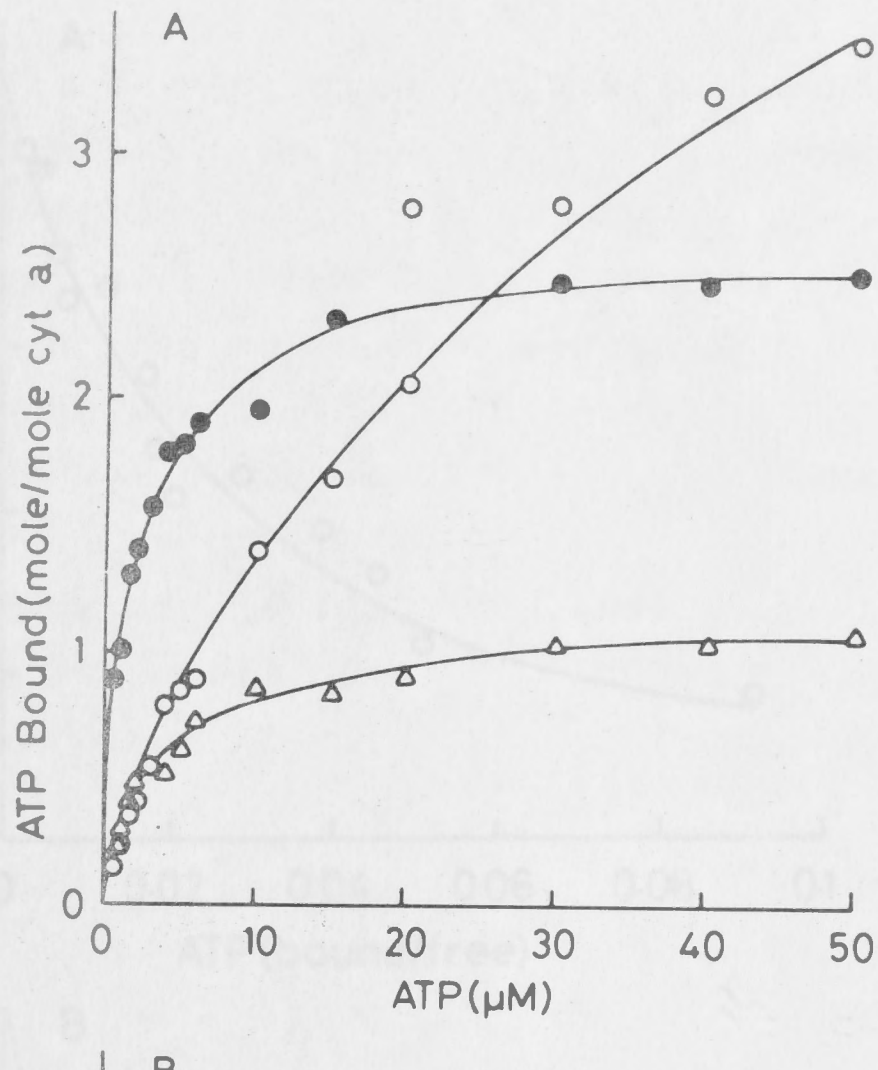

Figure 20. *Effect of phospholipase A treatment on the differentiation of ATP 'uptake' into specific binding, translocation and non-specific binding.*

The data in Figure 19 was recalculated as follows: specific binding (△) = difference between total uptake and ATP remaining in the pellet following atractyloside addition (i-iii); translocation (●) = difference in ATP uptake measured by adding atractyloside before and 2 minutes after the ATP (iii-ii); non-specific binding (○) = ATP uptake by the samples pretreated with atractyloside (ii). A, control mitochondria; B, venom phospholipase A treated mitochondria.

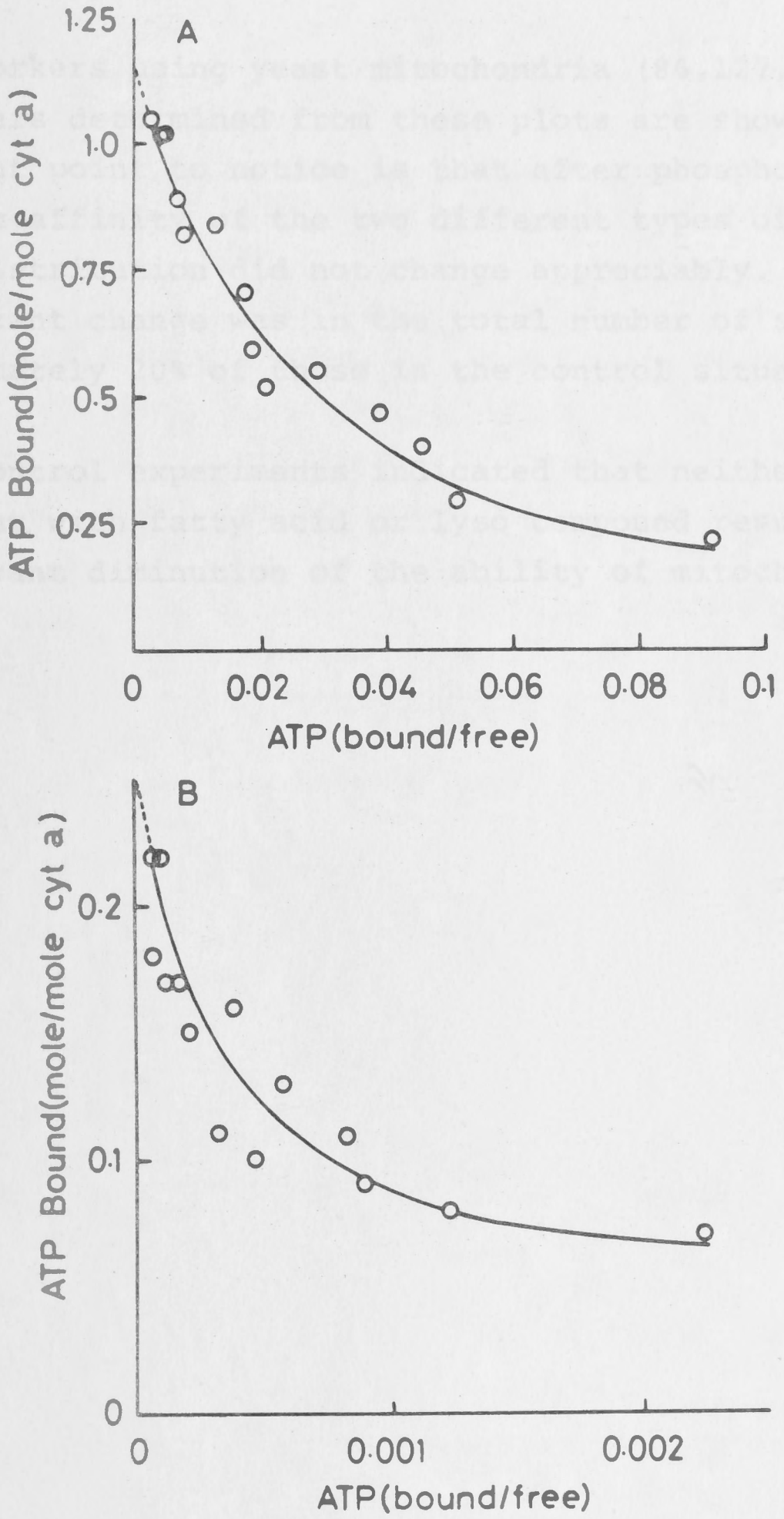

Figure 21. *Scatchard plots of atractyloside removeable binding of ATP to phospholipase A treated and control mitochondria.*

The experimental data derived from the differences in Figure 20 was recalculated and expressed as indicated by Scatchard (216).
A, control mitochondria; B, phospholipase A treated mitochondria.

other workers using yeast mitochondria (86,127,128). Binding parameters determined from these plots are shown in Table IV. The important point to notice is that after phospholipase A treatment both the affinity of the two different types of binding sites and their distribution did not change appreciably. The only significant change was in the total number of sites which fell to approximately 20% of those in the control situation.

Control experiments indicated that neither a BSA wash, nor treatment with fatty acid or lyso compound resulted in any significant diminution of the ability of mitochondria to bind ATP.

TABLE IV

Binding constants for atractyloside-removeable binding of ATP to phospholipase A treated mitochondria

The number of binding sites (C_o) and the dissociation constants (K_d) were determined from the Scatchard plots in Figure 21. Treatment of the experimental data is described in Section B. The cytochrome a concentration was 0·14μM for the control and 0·12μM for the phospholipase A treated mitochondria respectively.

Treatment	No. of sites mole/mole cyt a		Ratio high/low	Dissociation constants μM	
	C'_o	C''_o		K'_d	K''_d
Control	0.41	0.80	1:2	0.72	6.5
Phospholipase A treated	0.085	0.155	1:2	0.60	5.4

Discussion

This study provides new insights into the mechanism of adenine nucleotide translocation in rat liver mitochondria. The experiments presented demonstrate that a marked loss of adenine nucleotide translocase activity results when these mitochondria are partially depleted of their phospholipid. The use of phospholipases to deplete the mitochondria of their phospholipid proved to be most advantageous since it was possible to effectively control the extent and specificity of this depletion. The loss in ability of mitochondria to translocate adenine nucleotides could then be directly correlated with the loss in specific membrane phospholipids. Moreover, it was possible to obtain information about the way in which certain effectors such as metal ions and uncouplers influence translocation of adenine nucleotides in intact mitochondria.

Translocation of adenine nucleotides

The first point brought out in the data is that mitochondria need lose only a very small fraction of their phospholipid complement before incurring a considerable loss in ability to translocate ADP and ATP. For example, treatment of rat liver mitochondria with pancreatic phospholipase for 20 sec resulted in the removal of only about 1% of the total mitochondrial phospholipid yet the translocation of ADP was reduced some 50%. With venom-treated mitochondria a 50% decrease in ADP translocation was achieved after only 8% of the total phospholipid had been removed.

The second point is that ADP translocation is considerably more susceptible to phospholipid depletion than is ATP translocation. Thus treatment of rat liver mitochondria with sufficient phospholipase to bring about a 50% decline in ADP translocation, resulted in a loss of only 25% of ATP translocation (see Figures 7 and 8). Moreover, the rate of decay of ADP translocation was found to follow second order kinetics whereas that of ATP translocation followed first order kinetics.

It was consistently observed that the hydrolytic action of pancreatic phospholipase was more effective than that of the venom enzyme in reducing the ability of the mitochondria to translocate adenine nucleotides. Thus after 20 sec of treatment of rat liver mitochondria with pancreatic phospholipase when ADP and ATP translocation were diminished 50% and 25% respectively, 8%, 2% and 0% of the cardiolipin, phosphatidyl ethanolamine and phosphatidyl choline respectively, had been lost. In contrast, after 1 minute of treatment with venom phospholipase, when mitochondria have lost 50 and 25% of their ability to translocate ADP and ATP respectively, about 10% of the phosphatidyl ethanolamine and 10% of the phosphatidyl choline are lost from the membrane. No cardiolipin was lost in the same period of treatment. It is concluded from these findings that phosphatidylcholine is probably not involved in the translocation of adenine nucleotides *in situ*. On the other hand, it would seem that phosphatidylethanolamine and cardiolipin are obligatory components in the overall translocation system.

The ability of Ca^{2+} to stimulate translocation of ATP was progressively reduced with increasing times of treatment of rat liver mitochondria with the pancreatic phospholipase. Since this change in pattern was not observed on treatment of mitochondria with the venom enzyme it would seem that cardiolipin is necessary in order for Ca^{2+} to bring about stimulation of ATP translocation in intact mitochondria. This conclusion would be consistent with the well-known ability of cardiolipin to bind Ca^{2+} as observed in phospholipid bilayers (see ref. 189). Similarly, it is likely that interactions of La^{3+} with cardiolipin prohibits Ca^{2+} stimulation of ATP translocation (Section C) and potentiates Ca^{2+} stimulation of ADP translocation.

Cardiolipin also seems to be an important component of the system which allows uncouplers of oxidative phosphorylation to stimulate ATP translocation. In Table V are summarised proposed views concerning the involvement of the various phospholipids in the translocation of ADP and of ATP by rat liver mitochondria.

It could be argued that the effects observed after phospholipase treatment are an artefact produced by the products of

TABLE V

Summary of proposed involvement of phospholipids in adenine nucleotide translocation in rat liver mitochondria

	Function	Phospholipid requirement	Evidence
i	Translocation of ADP and ATP	CL>PE>PC	Much larger degree of hydrolysis of PE and PC required for equivalent loss in activity.
ii	Ca^{2+} stimulation	CL>PE>PC	Loss of stimulation of ATP translocation on loss of CL, PE. Strange behaviour of ADP after loss of CL.
iii	K^{+} stimulation	PE>PC,CL	Similar results obtained with both phospholipases.
iv	CCP stimulation of ADP inhibition	PE>PC,CL	Occurs with no hydrolysis of PC or CL
v	CCP inhibition of ATP	CL>PE>PC	Time lag seen with venom phospholipase.
vi	Mg^{2+} stimulation	CL, PE>PC	Loss of PC results in increased affinity.
vii	La^{3+} inhibition of Ca^{2+}-stimulation of ATP translocation.	CL>PE>PC	As for (ii).
viii	La^{3+} stimulation of ADP translocation.	Unclear	

CL, Cardiolipin; PE, phosphatidylethanolamine; PC, phosphatidylcholine.

phospholipid hydrolysis. Indeed, both fatty acids and lysophospholipids are inhibitors of adenine nucleotide translocation. The inhibition by fatty acids is thought to be dependent on the formation of fatty acyl CoA derivatives (157,229,251). For the following reasons it is argued that the primary effect of the phospholipases on the loss of translocation activity is due to loss of mitochondrial phospholipids. Firstly, all incubations were performed in the presence of and the mitochondria were washed with BSA. These conditions allow for binding of the fatty acids and lysocompounds to the BSA and their subsequent removal from the incubation medium. Secondly, quite different effects on the translocase were observed after depletion of the mitochondria of different (specific) phospholipids. These differences could not be reproduced by the addition of either fatty acids and/or lysophosphatidyl ethanolamine or lysophosphatidyl choline. Thirdly, there is no reversal of the CCCP inhibition of ADP translocation on the addition of any of the above compounds. Fourthly, fatty acid and lysophospholipid treatment of mitochondria results in a potentiation of the Ca^{2+} stimulation of ATP translocation whilst phospholipase action either inhibits this or has little effect.

Membrane structure

Studies with phospholipid-specific phospholipases yield important clues as to the spatial distribution of phospholipids within the membrane and their importance with respect to membrane structure and viability.

Hypotonic swelling of erythrocyte membranes is a prerequisite for phospholipid hydrolysis using either phospholipase A or C (137,275). These findings have been discussed in terms of masking of the phospholipid either by other membrane components, such as glycoproteins, or by their distribution on the inside of the membrane. A similar type of interaction may be invoked to explain the second phase of pancreatic phospholipase A induced phospholipid hydrolysis (Figure 1); after a certain proportion of the phospholipids have been hydrolysed and removed from the membrane the mitochondria begin to swell (cf. Figures 1 and 2) which exposes other phospholipid molecules to the hydrolytic enzyme. The fast second phase of phospholipid hydrolysis is not observed with the venom phospholipase A and provides more evidence for the important

role played by cardiolipin in mitochondrial integrity. In relation to this point a large proportion of the cellular cardiolipin is localised within the mitochondria (45), a not unreasonable observation when one considers that the necessary biosynthetic enzymes are also confined to these organelles (104,105).

Another obvious difference between the phospholipid hydrolysis curves is that the venom phospholipase A induced hydrolysis does not reach or even approach 100% (or if the contribution of cardiolipin is taken into account 85%). Again this may be associated with the accessibility of the enzyme to the substrate due to the non-hydrolysis of the cardiolipin. Another explanation is that product inhibition is involved but this is difficult to reconcile with the fact that (a) BSA is present to extract the hydrolysis products, and (b) the activity of another venom phospholipase A, that from *Naja naja*, is stimulated in the presence of fatty acids and BSA (233).

As for the leakage of endogenous adenine nucleotides and the inhibition of translocation, the pancreatic phospholipase A induced a faster rate of swelling of rat liver mitochondria. This result is not consistent, however, with the observation that lysophosphatidylcholine, the major product of the venom enzyme, is more effective than lysophosphatidylethanolamine, which is produced in large amounts by both enzymes, in disrupting the membrane integrity. From these results one could surmise that the actual depletion of the phospholipids from the mitochondria is the major determinant of membrane 'lysis' and consequent increased permeability.

Partial reversibility of fatty acid-induced swelling and leakage of the mitochondrial membranes is difficult to explain in view of the known lytic properties of these compounds (101,129,145,274). One possible explanation is that although fatty acids are lytic in nature they still have 'substance' and are able to 'plug' holes, albeit inefficiently, which appear in the membrane matrix. This hypothesis is consistent with results shown in Figure 5 where membranes that have been extracted with BSA tend to be more leaky, after the longer times of incubation with phospholipase A, than those that have not been treated in this manner.

Binding of adenine nucleotides

One might expect phospholipase A treatment to inhibit the translocation of adenine nucleotides by interferring with the transport of the carrier molecule through the membrane matrix. In view of this the decreased binding capabilities of phospholipase treated mitochondria are difficult to interpret. Phospholipid depletion may be responsible for the latter in two ways: (a) the binding reaction is dependent on phospholipids i.e. the conformation/structure of the membrane. There is a precedent for this supposition in that there exists a close relationship between structure-function relationships and the adenine nucleotide translocase as evidenced by the observation that the binding of adenine nucleotides to the translocase induces contraction of the inner mitochondrial membrane and decreased permeability properties (185,241,242). The reverse situation may well be operative in that the proper functioning of the translocase, in terms of binding capabilities, is dependent on the arrangement of the phospholipids within the membrane proper, (b) atractyloside is unable to remove ATP bound at the carrier site after phospholipase depletion either because the atractyloside binding sites are inactivated or the inhibitory action of atractyloside has been lost. In relation to the former Vignais *et al* (26)) have recently reported that both the binding and cooperativity of binding of carboxyatractyloside to the mitochondrial membrane is reduced by lipid depletion using aqueous acetone. The extent of binding may. however, be restored in the presence of a small amount of ADP. Interactions between atractyloside and binding of adenine nucleotides is very sensitive to the structure of the mitochondrial membrane as seen by the fact that specific binding of ADP, defined as the atractyloside-removeable portion, is abolished by detergent treatment and sonication. However, although the specific binding decreased there was a commensurate increase in unspecific binding after sonication. Sonication does not decrease atractyloside binding to the mitochondrial membranes (124). Similar increases in unspecific binding are not observed after phospholipase A treatment and indeed the unspecific binding component falls, albeit by a small percentage which could be correlated with loss of intramitochondrial sucrose-accessible space. This indicates that the loss in ATP binding is real and does not represent an

artefact produced by the atractyloside being unable to remove the bound adenine nucleotide. Two possibilities then exist regarding the fate of the ATP binding: (a) the active, or binding site, of the translocase enzyme is inactivated perhaps by a conformational change, and/or (b) the translocase enzyme itself has been lost from the membrane due to the phospholipid depletion. To distinguish between (a) and (b) binding assays would have to be carried out on the supernatant after phospholipase digestion assuming, of course, that the solubilised enzyme retains its binding properties and also as a second criteria, its atractyloside sensitivity. In view of the ready loss of the latter property upon for example, sonication, it is doubtful whether this could be met in a solubilised enzyme.

SUMMARY

1 Rat liver mitochondria were partially depleted of their phospholipids using phospholipase A prepared from porcine pancreas (substrate specificity, cardiolipin > phosphatidylethanolamine > phosphatidylcholine) or from *Crotalus adamanteus* venom (substrate specificity, phosphatidylethanolamine ∿ phosphatidylcholine > cardiolipin).

2 Removal of only about 1% of the mitochondrial phospholipid with the pancreatic enzyme leads to 50% and 25% losses in ADP and ATP translocation, respectively. Concomitant with the loss in adenine nucleotide translocation is a decline in the ability of both CCCP and Ca^{2+} to stimulate ATP translocation and of La^{3+} to inhibit Ca^{2+}-stimulated ATP translocation.

3 To achieve comparable losses in ADP and ATP translocation with the venom enzyme, it is necessary to remove about 8% of the total mitochondrial phospholipid. Following such treatment, CCCP and Ca^{2+} are still capable of stimulating ATP translocation. The ability of La^{3+} to inhibit Ca^{2+}-stimulated ATP translocation is reduced but not as greatly as after treatment with the pancreatic enzyme.

4 Control experiments involving treatment of the mitochondria with the products of phospholipase A digestion indicate that the effects observed on the translocase reflect a loss of phospholipid from the membrane.

5 It is concluded that phosphatidylethanolamine and cardiolipin but not phosphatidylcholine are necessary for the adenine nucleotide translocase to function in rat liver mitochondria and that cardiolipin is probably involved in the mechanism of CCCP and Ca^{2+} stimulation of ATP translocation.

6 Differences in the rates of swelling induced by pancreatic phospholipase A and venom phospholipase A indicate an important role for cardiolipin not only in the translocation process but also in overall mitochondrial integrity.

7 Binding studies indicate that there is a loss of the ability of venom phospholipase A treated mitochondria to bind ATP at atractyloside-removeable sites. The total number of sites decreases from 1·21 mole/mole of cytochrome a (the ratio of high to low affinity types is 1·2) to 0·24 mole/mole of cytochrome a

(high/low remains at approximately 1·2). The dissociation constants are 0·72μM and 6·5μM in the control mitochondria and 0·65μM and 5·4μM in the phospholipase A treated mitochondria.

8 Low concentrations of oleic acid induce swelling and leakage of endogenous adenine nucleotides from mitochondria. These phenomena are partially reversed by the use of higher concentrations of the acid. Higher concentrations still induce an irreversible lysis of the organelles.

SECTION F: CONCLUDING DISCUSSION

Control of adenine nucleotide translocation

Three ways by which adenine nucleotide translocation may be 'controlled' have been reported in the literature: (a) Shug *et al* (230) have reported a slow respiratory response to the addition of ADP to liver mitochondria from alloxan-diabetic rats and hibernating ground squirrels. This along with the observation that oleoyl-CoA inhibited adenine nucleotide translocation in isolated liver mitochondria *in vitro* lead to the proposal that certain long-chain fatty acid esters which accumulate in the liver during periods of excessive fatty acid oxidation act as natural regulators of adenine nucleotide translocation. Confirmatory experiments indicated that this hypothesis was indeed correct (149b). In this way the fatty acid ester content of the cell not only influences the translocation of adenine nucleotides but in doing so modulates respiration and overall mitochondrial metabolism. In relation to this McLean *et al* (157) have found that the equilibrium which is present between the mitochondrial and cytoplasmic compartments is lost in animals which had been treated so as to induce a high liver fatty acid ester concentration. A similar type of inhibition could be invoked to explain the results obtained by Pedersen and Gray (194) using brown adipose tissue mitochondria where a high ADP concentration was found to be necessary to achieve a state 4/state 3 respiratory change. This has been confirmed by later work of Christiansen *et al* (43) who have also proposed a role for the adenine nucleotide translocase in controlling thermogenesis in adipose tissue. (b) Babior *et al*(8) have reported an approximate 30-50% increase in the rate of translocation of ADP in liver mitochondria from rats which have been treated with thyroid hormones (triiodothyronine or thyroxine). ATP translocation under similar conditions was not tested. It is suggested that the increased rate of ADP translocation, which was accompanied by an increased rate of state 3 respiration, would lead to a buildup in the energy charge of the cell and a consequent increase in the activity of enzymes which utilise ATP. The latter may be mediated either *via* a direct effect of the energy charge on existing enzymes (2,3) or an actual increase in the amount of enzyme present in the cell (e.g. 108,109). Presumably the increased rate of translocation

of ADP is mediated *via* the production of a higher membrane concentration of the translocase enzyme as *in vitro* application of thyroid hormones at comparable concentrations has no effect on the rate of translocation. (c) Meisner (165) has suggested that changes in the cation environment of the mitochondrion, particularly as in the level of Ca^{2+} ion may be capable of influencing translocation rates in the physiological state. This statement was made, however, with the reservation that due to binding of metal ions to proteins and other anionic sites the 'free' concentration of cations in the cell would be significantly lower than that of the total (48,50). Similar conclusions have been drawn from results presented in Section C of this thesis. There must, however, be conditions in the cell during which the cellular concentration of Ca^{2+} ion would be sufficiently high enough to be able to modulate adenine nucleotide translocation.

Ca^{2+} and fatty acid esters and Ca^{2+} and thyroid hormones would be expected to exert some degree of fine control over adenine nucleotide translocation by acting as complementary effectors. In the case of the former pair this antagonism has been demonstrated by data presented in Section E, Figure 15.

A potential non-physiological means by which the adenine nucleotide translocase may be controlled is by way of drug action. Likely candidates for this would be local anesthetics, especially butacaine. The toxic effects of butacaine on living tissue have been discussed previously in terms of its observed inhibitory properties on the translocation process (Section E). Definite evidence to support (a) and (b) under *in vivo* conditions is forthcoming. The influence of butacaine would be relatively easy to test presuming that the butacaine which was administered to the animal remained bound at the membranes during the isolation procedure. However, technical difficulties alone preclude (c) from being substantiated; Ca^{2+} found associated with mitochondria is lost during isolation procedures due most probably to its low binding affinity and the presence of some type of metal chelator in most isolation media.

As a point of interest the effects mediated by (a), (b) and (c) are all under hormonal control; (a) by hormones involved in

the stimulation of fatty acid oxidation during starvation and diabetes, (b) obviously by thyroid hormones and (c) by hormones which are capable of raising cellular Ca^{2+} levels by acting on adenyl cyclase, e.g. glucagon in the liver.

Mechanism of adenine nucleotide translocation and nature of the translocase molecule

Differences between ATP and ADP translocation

A large variety of effectors including ions and membrane-active agents have opposite effects on or different degrees of influence on the translocation of ATP or ADP. Some of these effectors and their differences are listed in Table I.

It is difficult to equate the large number of different responses of ATP or ADP translocation only on the basis of a charge difference, i.e. the 3-ve charges of ADP as opposed to the 4-ve charges of ATP. One explanation for these discrepancies and which has been tentatively mentioned previously (Section D, discussion) is that there are two types of translocase molecules, one specific for ATP and the other for ADP. The observation that (a) there are two different types of binding sites for either ATP or ADP in mitochondria (267, Section D), and (b) the translocation process exhibits both a high and a low affinity for adenine nucleotides (201, Sections C,D and E) indicative of two classes of translocation sites lends credence to this hypothesis.

Another alternative which was mooted was that there are two separate translocation molecules one specific for ATP and the other for ADP. On the basis of this hypothesis it is, however, difficult to explain (a) the observed competition between ATP and ADP for both binding (267) and translocation (62,201,270, Section C), and (b) the *pro-rata* loss of low and high affinity adenine nucleotide binding sites after phospholipase treatment.

There is considerable evidence for a multi-sited adenine nucleotide translocase molecule comprised of an oligomeric structure including, as mentioned above, the two types of binding

TABLE I

Influence of effectors on the translocation of ATP and ADP

Effector	ATP	ADP
Ca^{2+}	large stimulation	small stimulation
- after pancreatic PLase treatment	small stimulation; higher K_a for Ca^{2+}	large stimulation; lower K_a for Ca^{2+}
- La^{3+} present	inhibition	stimulation
- local anesthetics	inhibition	stimulation → inhibition
CCCP	stimulation	inhibition
- after PLase treatment	decreased stimulation	stimulation
U^{2+}	M-M inhibition	Sigmoidal inhibition
La^{3+}	no effect	stimulation
Mg^{2+}	stimulates ATP to a lesser extent than ADP	
Lyso compounds	greater inhibition of ATP translocation	
- after BSA treatment	ATP more resistant to reversal than ADP	

Small differences were also noted with K^+ and membrane-active agents, etc. which are too numerous to mention.

M-M refers to Michaelis-Menton.

sites and two K_m values for translocation as well as (a) cooperative binding of the inhibitor carboxyatractyloside, and (b) the loss of binding after detergent treatment or sonication of the mitochondria. The latter would disrupt certain types of bonds holding the complex in the 'correct' conformation and result in altered binding 'capacity' although the number of sites remains constant (267).

At least three different types of mechanisms and variations thereon have been proposed to explain the action of translocating enzymes (see for example reference 176). They are (a) spatial movement of transporter molecules themselves across the membrane matrix, (b) rotation of the carrier in the membrane, and (c) provision of a pore in the membrane along whose inside the molecules to be transported are preferentially passed. Evidence accumulated with the local anesthetics, especially butacaine, would suggest that the hydrophobic interior of membrane is very important with respect to the adenine nucleotide translocation process but this still does not let one distinguish between the above possibilities. A clue to this dilemma, however, may be gleaned from the observation that adenine nucleotide translocation is an obligatory one-for-one exchange process. Thus (b), as suggested by Pardee (190) and Yariv *et al* (278), and consistent with the fluid mosaic model of membranes of Singer (232), would be favoured where there are binding sites on the inner and outer surfaces of the inner mitochondrial membrane both of which must be 'loaded' with adenine nucleotides for translocation to be effective and operative. Obligatory exchange of endogenous and exogenous adenine nucleotides is much more difficult to explain especially in terms of (c). In order to completely span the width of the mitochondrial membrane, in keeping with the rotatory carrier model, the translocase molecule would have to be approximately 80-100 Å across at least in one dimension. This would require a molecular weight of at least 250,000 daltons a not unreasonable value when one considers that the transporter itself probably has an oligomeric structure. Another transport protein, that of the Na^+,K^+-ATPase from beef brain, has a molecular weight of 670,000 daltons (217).

One shortcoming with previous models (267) of the translocase with regard to the distribution of low and high affinity binding sites (i.e. the high on the outside and the low on the inside) is that available evidence suggests that atractyloside is unable to cross the inner mitochondrial membrane, a necessary prerequisite in the binding assay employed (267, Section E).

It is necessary then to produce a model which would account for: (a) the observed ratio of high/low affinity adenine nucleotide binding sites, (b) the existence of two 'types' of translocation process, (c) the obligatory one-for-one exchange properties of the translocase. The binding/translocation sites on the outside of the membrane are thus duplicated on the inside, (d) different properties of ATP and ADP translocation (see Table I), and (e) the small (compared to that of ATP) stimulation by Ca^{2+} ions of ADP translocation.

These requirements lead to the model which is shown in Figure 1. It is suggested that ATP is preferentially bound at and translocated by a specific site of high affinity whilst it is bound at and translocated less efficiently by a low affinity site which possesses a high affinity with respect to ADP and *vice versa*.

To explain the observed 1:2 ratio of high/low affinity binding sites it is further proposed that one (or more) of the low affinity sites is common both for low affinity ATP and ADP binding. This site acts either as an effector site and/or an extra translocation site should the demand on the other sites become excessive. In view of the importance to the cell of adenine nucleotide translocation across the inner mitochondrial membrane it is quite conceivable that there would exist a regulatory site on the mitochondrion whose function it is to modulate this process.

Stimulation of adenine nucleotide translocation by Ca^{2+} is envisaged to occur at one site only, that being the site which has high affinity for ATP. This then explains the reduced degree of stimulation of ADP translocation as this molecule is suggested to be translocated at a reduced rate by this particular site.

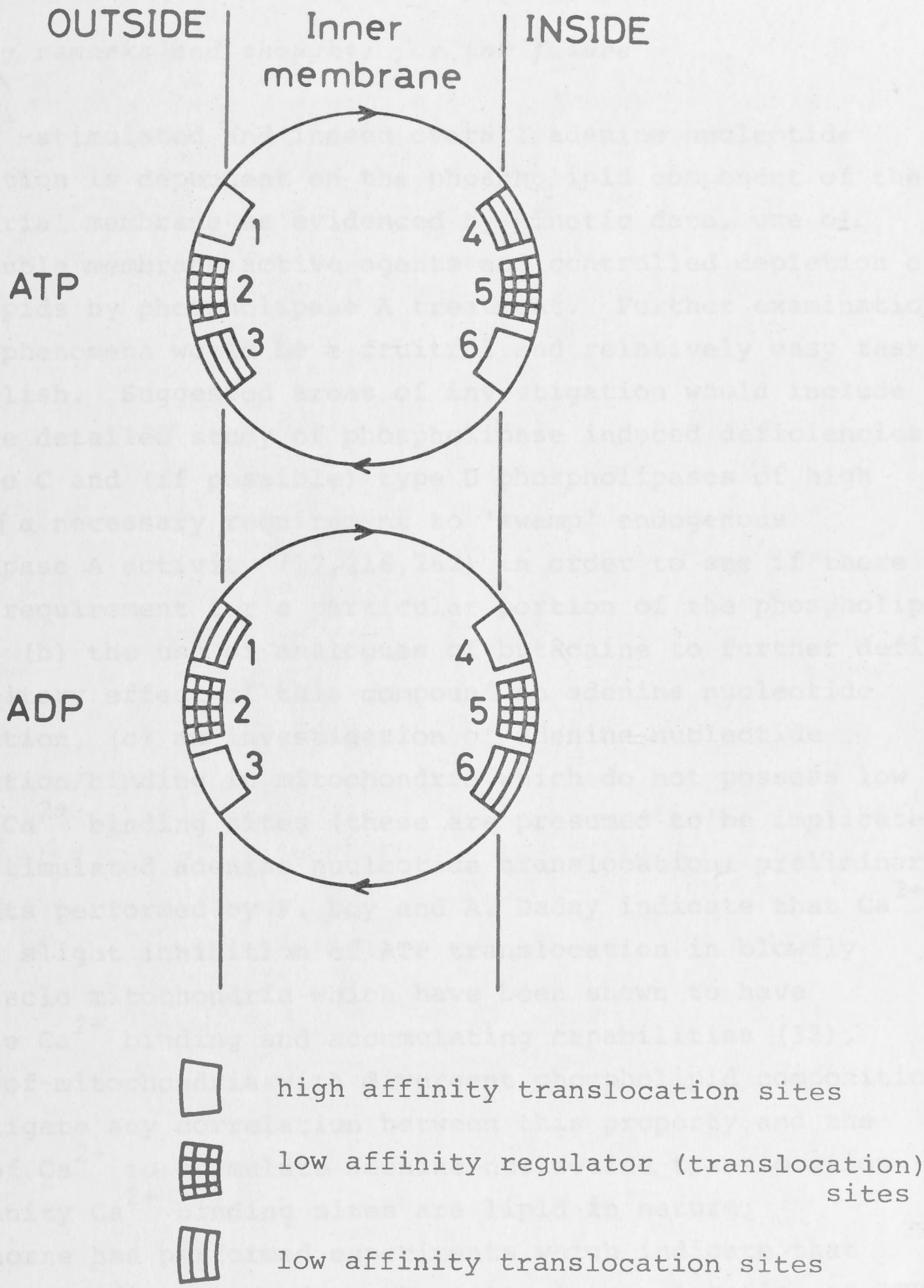

Figure 1. *Proposed model of the spatial distribution of adenine nucleotide binding sites on the translocase molecule and their specificity and affinity.*

See text for more details

Concluding remarks and thoughts for the future

Ca^{2+}-stimulated and indeed overall adenine nucleotide translocation is dependent on the phospholipid component of the mitochondrial membrane as evidenced by kinetic data, use of lipid-soluble membrane-active agents and controlled depletion of phospholipids by phospholipase A treatment. Further examination of these phenomena would be a fruitful and relatively easy task to accomplish. Suggested areas of investigation would include (a) a more detailed study of phospholipase induced deficiencies using type C and (if possible) type D phospholipases of high activity [a necessary requirement to 'swamp' endogenous phospholipase A activity (17,216,262) in order to see if there exists a requirement for a particular portion of the phospholipid molecule] (b) the use of analogues of butacaine to further define the inhibitory effect of this compound on adenine nucleotide translocation, (c) an investigation of adenine nucleotide translocation/binding in mitochondria which do not possess low affinity Ca^{2+} binding sites (these are presumed to be implicated in Ca^{2+}-stimulated adenine nucleotide translocation; preliminary experiments performed by F. Doy and A. Daday indicate that Ca^{2+} induces a slight inhibition of ATP translocation in blowfly flight muscle mitochondria which have been shown to have negligible Ca^{2+} binding and accumulating capabilities (32), (d), use of mitochondria with divergent phospholipid compositions to investigate any correlation between this property and the ability of Ca^{2+} to stimulate adenine nucleotide translocation (low affinity Ca^{2+} binding sites are lipid in nature; F.R.W. Thorne has performed experiments which indicate that Ca^{2+} ions significantly reduce the rate of translocation of ATP in mitochondria from Ehrlich ascites tumour cells which have a relatively low proportion of acidic phospholipids (13), and (e) the study of yeast mitochondria as has already been performed in several laboratories (86,127,128,137b,195) whereby by utilising mutants and inhibitors of protein synthesis one may gain an insight, not only into aspects of the genetics of the adenine nucleotide translocase but also into its structure and mechanism of action.

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APPENDIX

All abbreviations are as indicated in the instructions to authors of the *Biochemical Journal*.

Other unusual abbreviations are as follows:

HEPES, N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid

CCCP, Carbonyl cyanide m-chlorophenyl hydrazone

Butyl-PBD, 2(4'-t-Butylphenyl)-5-(4''-biphenylyl)-1,3,4-oxadiazole

MES, 2(N-Morpholino(ethane sulphonic acid

EGTA, Ethyleneglycol-bis(β-aminoethyl ether)N,N'-tetraacetic acid

R_{act}, relative action; the ratio of the rate of adenine nucleotide translocation in the presence of effector to that observed in its absence

O.D., optical density.

K_i, concentration of inhibitor required for half-maximal inhibition

K_a, concentration of activator needed for half-maximal activation (in some cases K_a is referred to as apparent K_m).